

G. Thomas D.M. Sabatini  
M.N. Hall (Eds.)

# **TOR**

## **Target of Rapamycin**



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# TOR

## Target of Rapamycin

With 49 Figures and 7 Tables



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George Thomas  
Friedrich-Miescher-Institute, Maulbeerstrasse 66, 4058 Basel, Switzerland  
e-mail: gthomas@fmi.ch

David M. Sabatini, MD, PhD  
Whitehead Institute, 9 Cambridge Center, Cambridge, MA 02142, USA  
e-mail: sabatini@wi.mit.edu

Michael N. Hall  
Biozentrum, University of Basel, Klingelbergstrasse 70, 4056 Basel,  
Switzerland  
e-mail: M.Hall@unibas.ch

*Cover Illustration by Sonja Dames:*

*Structure of the ternary complex consisting of FKBP12 (blue ribbon), rapamycin (red space filling representation), and the FRB domain of human TOR (yellow ribbon). The figure is based on the crystal structure published by J. Choi, J. Chen, S.L. Schreiber, and J. Clardy (1996, Science 273: 239–242, ‘Structure of the FKBP12-Rapamycin Complex Interacting with the Binding Domain of Human FRAP’; PDB file: 1FAP).*

ISBN 978-3-642-62360-8      ISBN 978-3-642-18930-2 (eBook)  
DOI 10.1007/978-3-642-18930-2

Library of Congress Catalog Card Number 72-152360

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Originally published by Springer-Verlag Berlin Heidelberg New York in 2004

Softcover reprint of the hardcover 1st edition 2004

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Typesetting: Stürtz AG, Würzburg

Cover Design: Design & Production GmbH, Heidelberg

Production Editor: Angélique Gcouta, Berlin

Printed on acid-free paper

SPIN: 10908495

27/3020

5 4 3 2 1 0



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## Preface

As the role of TOR continues to unfold, it is clear that this atypical protein kinase occupies a central role in eukaryotic biology as an energy and nutrient effector. It is also clear that with the advent of metazoans, the ancient signal transduction pathway in which TOR resides has been integrated with those that arose later to control metabolism and growth throughout a multicellular organism. TOR was first identified in *Saccharomyces cerevisiae* in a genetic selection for mutants resistant to the cytostatic effects of rapamycin. Rapamycin is a natural metabolite produced by soil bacteria, *Streptomyces hygroscopicus*, isolated from a soil sample collected on Easter Island, which the natives refer to as Rapa Nui. Later, it was shown that rapamycin forms a complex with the immunophilin FKBP12, and this complex then binds and inhibits TOR. Initially, rapamycin was brought into the laboratory as a fungicide, but was rapidly set aside when found to have immunosuppressive activity. With the advent of transplantation therapy, however, it was eventually “rediscovered” and approved for clinical use as an immunosuppressant. Recent attention has focused on rapamycin and its analogues in the treatment of solid tumors, rheumatoid arthritis, and restenosis.

Here we have attempted to gather the leaders in the field of TOR research to review the latest findings from their specific areas of expertise, from nutrient sensing in single cell organisms to the role of TOR in pathogenic states. However, since the writing of the chapters herein, and reflecting the rapid pace of the TOR field, important new players have emerged in the TOR signal transduction pathway. The new players include the products of the two tumor suppressor genes *TSC1* and *TSC2*, the small GTPase Rheb, and AMP dependent protein kinase. Also new is the suggestion that TOR may link nutrients and ageing. These new advances and insights continue to add to the excitement surrounding the TOR field.

Finally, we wish to thank the authors, who took time from very busy schedules, to describe their research. We also thank our colleagues at Springer-Verlag, in particular Anne Clauss and Angélique Gcouta, who produced the book.

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# TOR: The First 10 Years

A. Lorberg · M. N. Hall

Division of Biochemistry, Biozentrum, University of Basel, Klingelbergstr. 70,  
4056, Basel, Switzerland

E-mail: M.Hall@unibas.ch

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**Abstract** TOR was discovered and christened 10 years ago. On the occasion of this anniversary, we revisit the discovery of TOR and chronicle subsequent breakthroughs in *S. cerevisiae* that contributed to an understanding of TOR function in yeast and higher eukaryotes. In particular, we discuss findings that led to the realization that the function of TOR is to control cell growth in response to nutrients.

## 1

### Introduction

TOR was discovered and christened 10 years ago (Heitman et al. 1991a; Kunz et al. 1993). On the occasion of this anniversary, we revisit the discovery of TOR and chronicle subsequent breakthroughs in *S. cerevisiae* that contributed to an understanding of TOR function in yeast and higher eukaryotes. In particular, we discuss findings that led to the realiza-



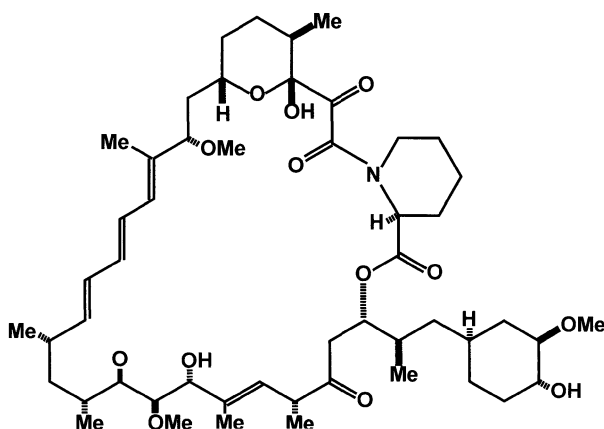


Fig. 1 Structure of rapamycin

tion that the function of TOR is to control cell growth in response to nutrients.

TOR is an acronym for “target of rapamycin”. Rapamycin, a lipophilic macrolide and secondary metabolite produced by the soil bacterium *Streptomyces hygroscopicus*, is a potent immunosuppressive, anticancer and antifungal drug (Fig. 1). The immunosuppressive activity of rapamycin is due to its ability to inhibit proliferation of helper T cells. Efforts to identify the cellular target of rapamycin and to unravel the drug’s mode of action were initiated in the late 1980s. One of the approaches exploited the antifungal activity of the drug. This approach made the assumption that the action and target of rapamycin are highly conserved—an assumption that proved to be correct.

## 2

### The Discovery of TOR

The early observation that rapamycin induces a G<sub>1</sub> arrest in *S.cerevisiae* cells as in T cells suggested that the drug’s target and mode of action are highly conserved, and that yeast could thus be used to study rapamycin action (Heitman et al. 1991a). To identify the target of rapamycin, spontaneous rapamycin-resistant yeast mutants were selected (Heitman et al. 1991a). Of 18 mutants isolated, 15 contained a loss-of-function mutation in the previously identified gene *FPR1* (FK506-binding proline rota-

mase), encoding the highly conserved cytoplasmic FKBP (FK506-binding protein) (Heitman et al. 1991a). The earlier cloning and targeted disruption of the *FPR1* gene in yeast had shown that FKBP is not essential for viability (Heitman et al. 1991b). Mammalian and yeast FKBP had been identified earlier also as an in vitro binding protein for rapamycin and FK506 (a potent immunosuppressant structurally related to rapamycin), and as a peptidyl-prolyl *cis-trans* isomerase (also known as a proline isomerase or rotamase) (Heitman et al. 1991b; Schreiber 1991). Because rapamycin was known to bind FKBP and to inhibit its isomerase activity in vitro, FKBP had been thought to be the target of rapamycin in vivo. However, the observation that targeted disruption of the *FPR1* gene did not affect viability (i.e., did not mimic rapamycin treatment) indicated that inhibition of FKBP was not the mechanism by which rapamycin inhibits growth (Heitman et al. 1991a,b). The apparent paradox of why inhibition of FKBP by mutation has no effect on viability whereas inhibition of FKBP by rapamycin inhibits growth was resolved by the observation that mutations in *FPR1* confer rapamycin resistance. The new and current model that emerged is that rapamycin binds FKBP and this complex then inhibits another target which is essential for growth or for an immune response (Bierer et al. 1990; Heitman et al. 1991a, 1992; Schreiber 1991). In other words, FKBP is a receptor required for drug action, but is not the growth-related target. The growth-related target of the FKBP-rapamycin complex was believed to be a component of a growth-controlling signal transduction pathway. The next step was to identify this mysterious signaling component.

The remaining three mutants from the original rapamycin resistance selection led to the identification of the FKBP-rapamycin target, which indeed turned out to be a novel signaling protein. The three remaining mutants were altered in one of two uncharacterized genes that were christened *TOR1* and *TOR2* (Heitman et al. 1991a). The three *TOR* mutants comprised two *TOR1* mutants (*TOR1-1* and *TOR1-2*) and one *TOR2* mutant (*TOR2-1*). Unlike the *FPR1* mutations, the mutations in *TOR1* and *TOR2* were gain-of-function mutations that conferred rapamycin resistance even in the presence of a wild-type, rapamycin-sensitive *TOR* allele.

The dominance of the rapamycin resistance-conferring *TOR* alleles was exploited to clone the *TOR* genes. Genomic libraries were constructed from the *TOR1-1* and *TOR2-1* mutants and used to transform wild-type yeast cells. The library members containing *TOR1-1* or *TOR2-1*

were selected based on their ability to confer rapamycin resistance on the sensitive wild-type strain (Helliwell et al. 1994, Kunz et al. 1993). Once the rapamycin-resistant mutant alleles of *TOR1* and *TOR2* were in hand, the rapamycin-sensitive, wild-type alleles were obtained by in vivo homologous recombination between a plasmid-borne mutant allele and a chromosomal wild-type allele (Helliwell et al. 1994, Kunz et al. 1993). The sequences of the *TOR1* and *TOR2* genes revealed that the encoded proteins are large (2470 and 2474 amino acids, respectively), highly similar (67% identical), and structurally related to phosphatidylinositol 3-kinase. TOR turned out to be the founding member of the phosphatidylinositol kinase-related protein kinase (PIKK) family (Keith and Schreiber 1995).

Shortly after the discovery of TOR in *S. cerevisiae*, the mammalian counterpart mTOR (also known as FRAP, RAFT, or RAPT) was identified based on its FKBP-rapamycin binding properties (Brown et al. 1994; Chiu et al. 1994; Sabatini et al. 1994; Sabers et al. 1995). To date, TOR has also been found in all other eukaryotes examined, including *S. pombe* (SpTOR; Kawai et al. 2001, Weisman and Choder 2001), *Cryptococcus neoformans* (CnTOR; Cruz et al. 1999), *Drosophila melanogaster* (dTOR; Oldham et al. 2000, Zhang et al. 2000), *Caenorhabditis elegans* (CeTOR; Long et al. 2002), and *Arabidopsis thaliana* (AtTOR; Menand et al. 2002). A comparative analysis of the different TOR proteins reveals that they are highly conserved (Table 1). Surprisingly, the presence of two homologous *TOR* genes seems to be unique to yeast and fungi (*S. cerevisiae*, *S. pombe*, and *C. neoformans*). Higher eukaryotes possess only one TOR gene. As described below, the two TORs in yeast are functionally similar, but not identical.

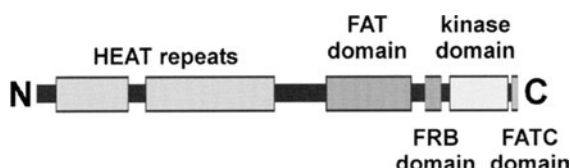
All TOR orthologues have a similar domain structure (Fig. 2). They contain a C-terminal domain that is highly homologous to the catalytic domain of phosphatidylinositol 3-kinase or phosphatidylinositol 4-kinase (Cafferkey et al. 1993; Kunz et al. 1993). However, none of the TORs has been shown to have lipid kinase activity; rather, the TORs display Ser/Thr-specific protein kinase activity. The catalytic domains of TOR1 and TOR2 are interchangeable, suggesting that the functional difference between TOR1 and TOR2 is not attributable to differences in catalytic activity (Helliwell et al. 1994). The catalytic domains of mTOR and yeast TOR are also interchangeable, and therefore also functionally equivalent (Alarcon et al. 1996). Next to the kinase domain is the FRB (FKBP-rapamycin binding) domain. This domain is the direct binding target of the



Table 1 Percentage of identity between TOR kinases from different organisms

Identity (%)									
	ScTOR1	ScTOR2	SpTOR1	SpTOR2	CnTOR1	CeTOR	AtTOR	dTOR	mTOR
ScTOR1	100	67	42	47	39	28	36	37	38
ScTOR2		100	43	48	40	28	38	38	40
SpTOR1			100	52	42	28	38	40	42
SpTOR2				100	44	29	42	42	44
CnTOR1					100	26	35	38	39
CeTOR						100	28	32	35
AtTOR							100	38	40
dTOR								100	53
mTOR									100

Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Cn, *Cryptococcus neoformans*; Ce, *Caenorhabditis elegans*; At, *Ara-*  
*bidopsis thaliana*; d, *Drosophila melanogaster*; m, mammalian (includes mouse, rat and human).



**Fig. 2** Domain architecture of TOR proteins. TOR proteins contain approximately 2,500 residues

FKBP-rapamycin complex. All mutations in *TOR* that confer rapamycin resistance affect this region and prevent the binding of the FKBP-rapamycin complex (Chen et al. 1995; Choi et al. 1996; Helliwell et al. 1994; Stan et al. 1994). The original rapamycin resistance-conferring *TOR1-1* and *TOR2-1* alleles contain the missense mutations Ser1972Arg and Ser1975Ile, respectively (Helliwell et al. 1994). Cafferkey et al. (1993) described rapamycin resistance-conferring *TOR1* (*DRR1*) alleles containing a Ser1972Arg or Ser1972Asn mutation. Recreating an analogous mutation in *mTOR* (Ser2035Ile) was instrumental in demonstrating that *mTOR* is the *in vivo* target of rapamycin in mammalian cells (Brown et al. 1995). Next to the FRB domain is the so-called FAT domain (Alarcon et al. 1999; Bosotti et al. 2000). This domain is found in all PIKKs and is always accompanied by a FATC domain at the extreme C-terminus of the protein. The FAT and FATC domains are speculated to mediate protein-protein interaction or to serve as a scaffold. The N-terminus of TOR contains two large blocks of HEAT repeats (originally found in huntingtin, elongation factor 3, the A subunit of protein phosphatase 2A, and TOR1) which are also commonly assumed to mediate protein-protein interactions. At least for TOR2, the HEAT repeats are required for localization of the protein to membranes (Kunz et al. 2000).

Although TOR1 and TOR2 are structurally and functionally similar, they are not functionally identical. Deletions of *TOR1* and *TOR2* confer different phenotypes (see below), and the N-terminal portions of the TOR proteins containing the HEAT repeats are not interchangeable. The HEAT repeats may confer specificity and thereby account for the functional difference between TOR1 and TOR2 (Helliwell et al. 1994). The functional difference between TOR1 and TOR2 is discussed in the following section.

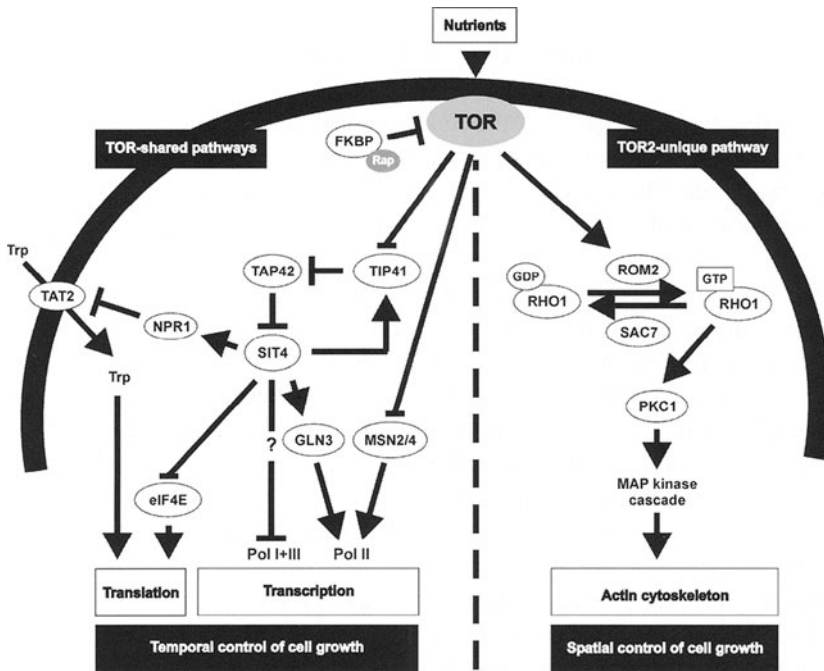
## 3

**TOR Controls Cell Growth in Response to Nutrients**

After the discovery of TOR, the next step was to elucidate its role in the cell. Results from a number of laboratories showed that rapamycin-treated or TOR-deficient yeast cells arrest growth and display several properties indicative of a general defect in mass accumulation, including an arrest in the G<sub>1</sub> phase of the cell cycle, downregulation of protein synthesis and ribosome biogenesis, changes in transcription, upregulation of mRNA degradation, induction of autophagy, and turnover of nutrient permeases (Albig and Decker 2001; Barbet et al. 1996; Beck et al. 1999; Heitman et al. 1991a; Noda and Ohsumi 1998; Powers and Walter 1999; Schmelzle and Hall 2000). The finding that the TOR proteins regulate a large variety of readouts that collectively determine the mass of the cell led to the conclusion that the role of TOR is to control cell growth, i.e., the increase in the dry weight of the cell (Schmelzle and Hall 2000).

We were originally misled by the rapamycin-induced G<sub>1</sub> arrest into believing that the primary role of TOR was to control the cell cycle. Only after further characterization of *tor* mutants, did we realize that the primary role of TOR is to control cell growth (Barbet et al. 1996; Thomas and Hall 1997). The observed cell cycle defect is a secondary consequence of a growth defect, i.e., cells need to grow to divide. However, the distraction of our early focus on the cell cycle was not fruitless, as it led us to explore the relationship between cell growth and cell division (Barbet et al. 1996).

The role of TOR as a controller of cell growth, like TOR itself, is conserved (Fingar et al. 2002; Menand et al. 2002; Oldham et al. 2000; Zhang et al. 2000). In higher eukaryotes, a TOR deficiency results in smaller cells, as observed for both dividing cells (Fingar et al. 2002; Kim et al. 2002) and postmitotic cells (Bodine et al. 2001; Shioi et al. 2002). A TOR deficiency in yeast, however, results in slightly larger cells (Kunz et al. 1993; Barbet et al. 1996), possibly because such cells have a swollen vacuole and an increase in wet weight that offsets the decrease in dry weight. An early hint that TOR controls cell growth (rather than the cell cycle) was the observation that a *tor* mutant cell does not increase in size as much as a *cdc28* mutant cell, a bona fide cell cycle mutant. *cdc28* cells arrest in G<sub>1</sub>, like *tor* cells, but then become very large due to ongoing cell growth in the absence of cell division. Thus, TOR-deficient yeast cells



**Fig. 3** TOR signal transduction pathways in yeast. The figure is incomplete as it illustrates only those aspects of TOR signaling discussed in this chapter. The TOR-shared pathways are mediated by TOR1 or TOR2 (TORC1). The TOR2-unique pathway is mediated only by TOR2 (TORC2). Only the TOR-shared pathways are rapamycin-sensitive (Zheng et al. 1995; Loewith et al. 2002)

are also smaller, but only when compared to an appropriate cell cycle mutant.

TOR controls cell growth in response to nutrients, including nitrogen and possibly carbon sources (Barbet et al. 1996; Schmidt et al. 1998; Beck and Hall 1999; Cardenas et al. 1999; Hardwick et al. 1999; Komeili et al. 2000; Crespo et al. 2002). The realization that TOR responds to nutrients stemmed from the finding that rapamycin-treated or TOR-deficient yeast cells are indistinguishable from starved cells (Barbet et al. 1996). Later, it was found that mTOR also responds to nutrients (Hara et al. 1998; Wang et al. 1998; Xu et al. 1998; Kimball and Jefferson 2002). Branched amino acids, leucine in particular, regulate the phosphorylation and thereby the inhibition of the eIF4E binding protein 4E-BP (also known as PHAS-I), and the phosphorylation and activation of p70<sup>S6k</sup>

(now known as S6K1 and S6K2), both downstream effectors of mTOR (Gingras et al. 2001, Dennis et al. 1999). The observation that dTOR-deficient flies appear starved provided further evidence that TOR responds to nutrients (Oldham et al. 2000; Zhang et al. 2000).

How do TOR1 and TOR2 control cell growth? Phenotypic analysis of *tor1* and *tor2* mutants revealed that the roles of TOR1 and TOR2 in growth control are different but overlapping. Deletion of *TOR1* causes only a mild phenotype, hypersensitivity to rapamycin and high temperature. In contrast, deletion of *TOR2* is lethal, causing cells to arrest throughout the cell cycle within approximately four generations and with a depolarized actin cytoskeleton. Finally, the simultaneous deletion of both genes is lethal and phenocopies the G<sub>1</sub> arrest that is observed upon rapamycin treatment (Barbet et al. 1996; Heitman et al. 1991a; Helliwell et al. 1994; Kunz et al. 1993). The growth arrest upon deletion of both *TOR* genes occurs within one generation. These findings suggested that the TOR proteins perform an overlapping function in rapamycin-sensitive signaling, but that at least TOR2 performs an additional, unique function. These two essential functions have been referred to as the “TOR-shared” and “TOR2-unique” functions (Helliwell et al. 1998a). The TOR-shared function mediates temporal control of cell growth, whereas the TOR2-unique function mediates spatial control of cell growth, each via different signaling pathways (see below; Fig. 3).

### 3.1

#### Temporal Control of Cell Growth

The TORs mediate temporal control of cell growth by positively regulating anabolic processes such as translation, transcription, and ribosome biogenesis, and by negatively regulating catabolic processes such as protein and RNA degradation, all in response to nutrients (Albig and Decker 2001; Dennis et al. 1999; Powers and Walter 1999; Schmelzle and Hall 2000). TOR controls many, if not most, of these processes via the type 2A-related phosphatase SIT4 (Beck and Hall 1999; Di Como and Arndt 1996; Schmidt et al. 1998). Under good nutrient conditions, TOR promotes the association of SIT4 with the TAP42 protein, and thereby maintains SIT4 inactive. Upon rapamycin treatment or nutrient limitation, SIT4 is released from TAP42 and activated. Activated SIT4, in turn, dephosphorylates a number of known and unknown distal TOR effector proteins. TOR may regulate the TAP42-SIT4 complex directly or via in-

hibition of a recently identified protein termed TIP41 (TAP42-interacting protein of 41 kDa; Jacinto et al. 2001; Jiang and Broach 1999). TIP41 binds TAP42 and promotes the dissociation of SIT4 from TAP42. TIP41 is a phosphoprotein that is dephosphorylated and activated by SIT4. Thus, TIP41 is part of a feedback loop the role of which is to rapidly amplify SIT4 phosphatase activity under TOR inactivating conditions (Fig. 3).

### 3.1.1

#### Translation Initiation

One of the processes regulated by the TOR-shared function is translation initiation, most likely cap-dependent translation initiation. Downregulation of translation initiation is among the earliest effects observed upon TOR inactivation (Barbet et al. 1996). This translation defect is the cause, indirectly, of the G<sub>1</sub> arrest observed upon rapamycin treatment. Evidence that the G<sub>1</sub> arrest is due to a deficiency in protein synthesis came from the finding that TOR- and cap-independent expression of the G<sub>1</sub> cyclin CLN3 is sufficient to suppress the rapamycin-induced G<sub>1</sub> arrest (but not the rapamycin-induced growth arrest) (Barbet et al. 1996). Hence, TOR-deficient cells arrest in G<sub>1</sub> because they are unable to synthesize the short-lived G<sub>1</sub> cyclins required for G<sub>1</sub> progression. TOR1 and TOR2, like mTOR, seem to activate translation initiation via the cap-binding initiation factor eIF4E (Barbet et al. 1996; Beretta et al. 1996; Cosentino et al. 2000). As described elsewhere in this volume (see chapters by Gingras et al. and Proud), the TORs and mTOR also control translation independently of eIF4E.

### 3.1.2

#### Protein Traffic and Degradation

The initial hint that TOR negatively controls protein degradation came from two observations (Beck et al. 1999; Schmidt et al. 1998). First, yeast strains prototrophic for amino acids are less sensitive to rapamycin. Second, rapamycin leads to a decrease in amino acid import. These observations suggested that TOR maintains amino acid permease activity. Indeed, TOR prevents at least the tryptophan transporter TAT2 from being targeted to the vacuole for degradation (Beck et al. 1999). TOR appears to prevent TAT2 degradation by inhibiting the SIT4-dependent dephos-

phorylation and activation of the protein kinase NPR1 (Jacinto et al. 2001; Schmidt et al. 1998). Activated NPR1 may directly phosphorylate and thereby induce the ubiquitination, internalization, and degradation of TAT2 (Beck et al. 1999; Schmidt et al. 1998; Fig. 3). As described elsewhere in this volume (see chapter by Kamada et al.), TOR also negatively controls autophagy, the vacuolar targeting and degradation of bulk cytoplasm, via phosphorylation and inhibition of the protein kinase APG1-APG13 (Kamada et al. 2000; Noda and Ohsumi 1998). The transport of bulk cytoplasm to the vacuole may account for the swollen vacuole observed in rapamycin-treated cells (see above). Interestingly, like TOR in yeast, mTOR also negatively regulates autophagy (Blommaert et al. 1995) and, as shown recently, maintains nutrient permeases (amino acid and glucose transporters) on the cell surface (Edinger and Thompson, 2002).

### 3.1.3

#### Nuclear Localization of Nutrient-Responsive Transcription Factors

TOR broadly controls transcription, both positively and negatively. Importantly, and as described elsewhere in this volume, TOR activates transcription of glycolysis, rRNA, tRNA, and ribosomal protein genes, all central to cell growth (Cardenas et al. 1999; Hardwick et al. 1999; Powers and Walter 1999; Zaragoza et al. 1998). The mechanisms by which TOR activates these Pol I-, Pol II- and Pol III-dependent genes are unknown. TOR inhibits transcription normally induced upon starvation or other types of stresses (Beck and Hall 1999; Crespo et al. 2001). For example, TOR inhibits transcription of genes activated upon nitrogen limitation by maintaining the GATA transcription factor GLN3 phosphorylated and thereby tethered to the cytoplasmic protein URE2 (Beck and Hall 1999). Upon TOR inactivation due to nitrogen limitation or rapamycin treatment, activated SIT4 dephosphorylates GLN3. Dephosphorylated GLN3 dissociates from URE2 and moves into the nucleus to activate target genes involved in the utilization of alternative nitrogen sources or in the synthesis of glutamine. GLN3 is activated specifically in response to depletion of the intracellular pool of glutamine, the preferred nitrogen source for yeast (Crespo et al. 2002). TOR also sequesters several other transcription factors, such as GAT1, MSN2, MSN4, RTG1, and RTG3, in the cytoplasm (Beck and Hall 1999; Komeili et al. 2000). These factors are also activated upon glutamine starvation (RTG1 and RTG3) or in re-

sponse to other types of nutrient or environmental stresses (GAT1, MSN2, and MSN4). Interestingly, TOR appears to discriminate between different nutrient conditions to elicit a response appropriate to a given condition (Beck and Hall 1999; Crespo et al. 2002; Kuruvilla et al. 2001; Kawai et al. 2001; Weisman and Choder 2001). The mechanisms by which TOR senses and discriminates between different nutrients and environmental stresses are also unknown.

## 3.2

### Spatial Control of Cell Growth

#### 3.2.1

##### Polarization of the Actin Cytoskeleton

Growth of a daughter yeast cell occurs at a specific site (the bud site) on the surface of a mother cell. This polarized growth pattern is determined by a similarly polarized actin cytoskeleton which orients the secretory pathway, and thus newly made proteins and lipids, toward the growth site. TOR2 mediates spatial control of cell growth by regulating the polarization of the actin cytoskeleton. The first indication that TOR2 is involved in regulating the actin cytoskeleton was the isolation of the gene *TCP20* (now named *CCT6*) as a multicopy suppressor of a TOR2 deficiency (Schmidt et al. 1996). *CCT6* is a component of the TCP-1 chaperonin complex involved in the folding of actin and tubulin. Direct examination of the actin cytoskeleton in a conditional *tor2* mutant revealed that TOR2 is required for the polarized organization of the actin cytoskeleton. Instead of the cell-cycle dependent polarization of the actin cytoskeleton observed in a wild-type strain, a *tor2* mutant exhibits a randomized actin cytoskeleton at all stages of the cell cycle (Schmidt et al. 1996). TOR in higher eukaryotes has not been demonstrated to have a role in the organization of the actin cytoskeleton. The lack of such a role for TOR in higher eukaryotes may explain why these organisms have only one TOR.

How does TOR2 signal to the actin cytoskeleton? The actin and growth defects of a *tor2* mutant are also suppressed by deletion of the *SAC7* gene, encoding a GTPase-activating protein that downregulates the small GTPase RHO1 (Schmidt et al. 1997). This finding led to the observation that any condition which increases the amount of GTP-bound RHO1 in the cell, such as overexpression of RHO1 exchange factors (ROM2 or TUS1) or of RHO1 itself, suppresses a *tor2* mutation (Bickle et al. 1998;



Schmelzle et al. 2002; Schmidt et al. 1997). Finally, it was shown that GDP/GTP exchange activity toward RHO1 is reduced in a cellular extract derived from a *tor2* mutant. These findings suggested that TOR2 signals to the actin cytoskeleton by activating the RHO1 GTPase switch (Fig. 3). Subsequent findings indicated that RHO1, in turn, signals to the actin cytoskeleton via its direct effector PKC1 and the PKC1-controlled MAP kinase module composed of BCK1 (MAPKKK), MKK1 and 2 (MAPKK), and MPK1/SLT2 (MAPK) (Helliwell et al. 1998b).

#### 4

### Two TOR Complexes

The many protein-protein interaction domains in TOR1 and TOR2 suggest that the TORs form large complexes. Indeed, as revealed by the recent purification of TOR1 and TOR2, the TORs are components of two functionally distinct multiprotein complexes (Loewith et al, 2002). TOR complex 1 (TORC1) contains TOR1 or TOR2, KOG1, and LST8. The previously uncharacterized protein KOG1 (kontroller of growth) was briefly called COG1, until we were asked by a consortium of yeast laboratories working on a complex of the Golgi to surrender the COG acronym. KOG1 and LST8 are conserved, and their mammalian counterparts, raptor (mKOG1) and mLST8, interact with mTOR (Kim et al. 2002; Hara et al. 2002; Loewith et al. 2002). TOR complex 2 (TORC2) contains TOR2, LST8, AVO3 (formerly named TSC11), and the two previously uncharacterized proteins AVO1 and AVO2 (adheres voraciously to TOR2). TORC1 disruption mimics rapamycin treatment, and TORC2 disruption mimics TOR2 depletion. FKBP-rapamycin binds TORC1 but not TORC2. These findings, consistent with the previous suggestion that only TOR-shared signaling is rapamycin sensitive (Zheng et al. 1995), indicated that TORC1 mediates TOR-shared signaling and TORC2 mediates TOR2-unique signaling (Loewith et al. 2002). Thus, the distinct TOR complexes account for the diversity, specificity, and selective rapamycin inhibition of TOR signaling.

TORC1 and possibly TORC2 are conserved from yeast to man (Kim et al. 2002; Hara et al. 2002; Loewith et al. 2002). Interestingly, should TORC2 be conserved, there may be rapamycin-insensitive signaling in mammals. Rapamycin-insensitive signaling in mammals has so far not been detected, possibly because studies on TOR signaling in mammalian cells have relied heavily, if not exclusively, on the use of rapamycin to inhibit mTOR.

## 5

**Conclusion**

Many questions remain. How does TOR sense and discriminate between different nutrients and environmental stresses? How does TOR control readouts such as Pol I- and Pol III-dependent transcription and ribosome biogenesis, among other readouts? What are the specificity determinants that dictate the composition and architecture of the TOR complexes? Are there other TOR complexes? What are the specific molecular functions of the individual TOR partner proteins within each TOR complex? How do the partner proteins relate to the various upstream cues and downstream effectors of TOR? To date, there is no confirmed direct substrate for TOR in yeast. What are these substrates and what is the consensus phosphorylation site for TOR? Importantly, how can the information obtained thus far be used to generate better immunosuppressive, anticancer, or other types of drugs (Huang and Houghton 2002)? The second decade promises to be as interesting as the first.

*Acknowledgment.* We wish to acknowledge the Schreiber laboratory which, unfortunately, is not represented in this volume, but which has played a major role in the first 10 years of the TOR/FRAP field. We also acknowledge support from the Federation of European Biochemical Societies (A. L.), and from the Canton of Basel and the Swiss National Science Foundation (M. N. H.).

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# The Role of Phosphatases in TOR Signaling in Yeast

K. Düvel · J. R. Broach

Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA  
E-mail: jbroach@molbio.princeton.edu

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**Abstract** The TOR pathway controls cellular functions necessary for cell growth and proliferation of yeast and larger eukaryotes. The search for members of the TOR signaling cascade in yeast led to the discovery of type 2A protein phosphatases (PP2A) as important players within the pathway. We describe the roles in yeast of PP2A and the closely related phosphatase, Sit4, and then focus on complexes formed between the catalytic subunit of these phosphatases and Tap42, a direct target of the Tor protein kinases in yeast. Recent results suggest that Tap42 mediates many of the Tor functions in yeast, especially those involved in transcriptional modulation. However, whether Tap42 executes its function by inhibiting phosphatase activity or by activating phosphatases is still uncertain. In addition, Tor affects some transcriptional and physiological processes through Tap42 independent pathways. Thus, Tor proteins use multiple mechanisms to regulate transcriptional and physiological processes in yeast.

## 1

**Introduction**

The TOR pathway controls cellular functions necessary for cell growth and proliferation of yeast and larger eukaryotes. Primarily, these Tor promoted events converge to facilitate efficient translation. In mammals, Tor enhances translation initiation by promoting phosphorylation both of p70<sup>S6</sup> kinase, which in turn phosphorylates ribosomal protein S6, and of 4E-BP1, which by phosphorylation is relieved of its inhibitory activity against eIF-4E. Since *Saccharomyces cerevisiae* lacks functional homologues of either p70<sup>S6</sup> kinase or 4E-BP1, Tor must promote translation in yeast by a different means. The search for members of the TOR signaling cascade in yeast led to the discovery of type 2A protein phosphatases (PP2A) as important players within the pathway. This review will briefly introduce PP2A and the closely related phosphatase, Sit4, and then focus on complexes formed between the catalytic subunit of these phosphatases and Tap42, a direct target of the Tor protein kinases in yeast. While these complexes clearly play a central role in the TOR signaling pathway in yeast, the nature of its activity and the consequences of its action are far from clear. In addition, the role of the mammalian homologue of Tap42,  $\alpha 4$  protein, in TOR signaling in metazoans remains unresolved. We will speculate on the possible function of this protein and suggest directions that need to be explored in order to better define the role of this critical factor in TOR signaling.

## 2

**The Structure and Function of Protein Phosphatase 2A in Yeast**

## 2.1

**Protein Phosphatase 2A (PP2A)**

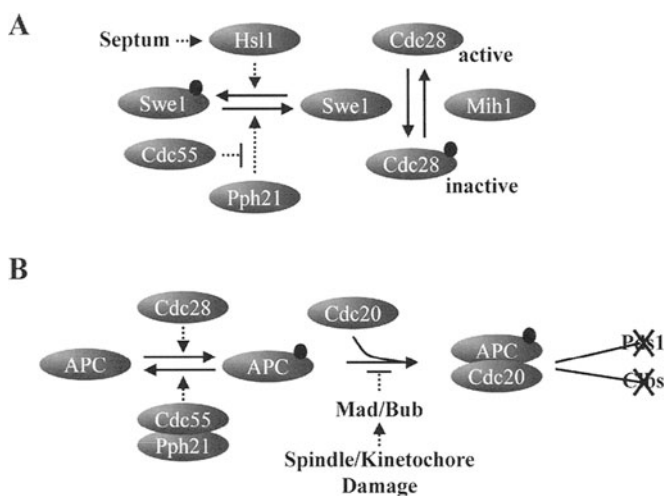
PP2A exists primarily as a heterotrimeric complex, consisting of a scaffold protein (the A subunit) on which sit the catalytic (C) subunit and either of two regulatory subunits, B or B'. The redundant genes *PPH21* and *PPH22* encode the C-subunit in *S. cerevisiae*. A third gene, *PPH3*, encodes a catalytic subunit of a protein phosphatase that can partially substitute for *PPH21* and *PPH22* and allows slow growth in a *pph21Δ pph22Δ* double deletion strain (Ronne et al. 1991). Conditional inactivation of the catalytic subunit yields cells arrested in growth with a 2N



DNA content and unable to enter mitosis. In addition, these mutants exhibit altered bud morphology and an abnormal actin cytoskeleton (Lin and Arndt 1995; Evans and Stark 1997). Similar defects arise from mutations of the regulatory subunits of PP2A (see below). In sum, these observations indicate that, as might be expected for a major cellular phosphatase, PP2A plays multiple roles in the cell, at least one of which is essential for growth.

Alternative regulatory subunits dictate the specificity of the catalytic subunit of PP2A. In yeast, two such subunits exist: *CDC55* codes for a protein with homology to the B family of mammalian regulatory subunits, while *RTS1* codes for a protein with homology to the B' family. No other proteins with homology to mammalian B subunits are encoded in the yeast genome.

Cdc55 plays a role in cell cycle progression, primarily through its effect on two cell cycle checkpoint systems. Cells deleted for *CDC55* grow normally at room temperature or higher but become elongated with multiple buds and aberrant nuclei when grown at lower temperatures (Healy et al. 1991). These phenotypes appear to result from effects of Cdc55 on a cell integrity checkpoint system. This system couples bud-neck septum formation to the activation of S-phase cyclins and the consequent progression into S phase as well as the morphological transition from apical to isotropic bud growth. Figure 1A provides a model summarizing a number of studies suggesting that this checkpoint involves inactivating, Swe1-dependent phosphorylation of Cdc28 on threonine-18/tyrosine-19. Swe1 can phosphorylate Cdc28 to inhibit its activity and this inhibition precludes activation of early cell-cycle events (Booher et al. 1993). In normal cells, formation of the septum activates the Hsl1 kinase, which phosphorylates Swe1, targeting it for ubiquitin-mediated degradation. In the absence of Cdc55, the unrestrained activity of Pph21/22 dephosphorylates and stabilizes Swe1, maintaining Cdc28 in its inactive form even in the presence of a functional septum. This results in elongated buds, incomplete DNA synthesis and attenuation of cell cycle progression. Consistent with the model in Fig. 1A: (1) a Cdc28<sup>Y19F</sup> allele suppresses the *cdc55* phenotypes, (2) *hsl1* mutants exhibit the same morphological and cell cycle defects as do *cdc55* mutants, and (3) deletion of *PPH21* and *PPH22* suppresses the *cdc55* phenotype (Yang et al., 2000). Significantly, these results suggest that in this case Cdc55 functions in a negative role, restraining Pph21/22 activity towards



**Fig. 1A, B** PP2A involvement in cell cycle checkpoints. **A** The cell integrity checkpoint. The model postulates that Cdc28 activity is inhibited by phosphorylation by Swe1-kinase, whose activity is reciprocally regulated by Hsl1 kinase and the PP2A catalytic subunit (*Pph21*). The PP2A catalytic activity is normally restricted by the Cdc55 regulatory subunit, allowing inactivation of Swe1 upon formation of a septum and consequent activation of Hsl1, resulting in active Cdc28 kinase and entry into S phase. In the absence of Cdc55 function, Swe1 remains active, thus blocking cell cycle progression, yielding elongated buds and incomplete DNA replication. **B** The spindle checkpoint. The model postulates that PP2A, directed by the Cdc55 regulatory subunit, counteracts a Cdc28-mediated activation of the APC required for binding Cdc20 and subsequent progression to anaphase. Loss of Cdc55 would yield hyperactivation of APC, potentially overriding spindle checkpoint signals transmitted by the Mad/Bub checkpoint pathway. *Black dots* represent phosphorylation

Swe1 rather than enhancing it. Thus, PP2A regulatory subunits can exert a critical inhibitory effect on phosphatase activity.

Cdc55 also plays a role in metaphase/anaphase transition, likely through regulation of the anaphase promoting complex (APC) activity. *cdc55* mutants fail to prevent sister chromatid separation in cells with defective mitotic spindles or kinetochores (Minshull et al. 1996; Wang and Burke 1997). However, unlike other spindle checkpoint mutants such as those defective in *BUB* or *MAD* genes, *cdc55* cells retain high Cdk activity under the arrest-inducing conditions. Figure 1B suggests a mechanism that could account for the observations regarding Cdc55 and the spindle checkpoint. In particular, this model postulates that

phosphorylation of APC by Cdc28 promotes interaction with the APC regulatory subunit, Cdc20, an effect counteracted by Cdc55-targeted PP2A activity. Cdc20 normally directs APC ubiquitin-conjugating activity to Pds1 and S-phase cyclins, leading to sister chromatid separation and the subsequent loss of Cdk activity that promotes exit from mitosis. In this model, loss of Cdc55 would increase the pool of phosphorylated APC, driving APC-Cdc20 complex formation and partially overriding Bub/Mad-inhibition of complex formation. Consistent with this model, deletion of *CDC55* suppresses the growth defect of *cdc20* alleles (Wang and Burke 1997). In addition, adenovirus E2orf4 inhibits yeast cell growth by inactivating APC in a Cdc55-dependent fashion and promoting PP2A interaction with APC (Kornitzer et al. 2001). Although this is a heterologous interaction, it demonstrates the capability of Cdc55-regulated PP2A activity to affect APC. Consistent with the proposed model, reduced Cdk activity exacerbates the effect of E2orf4 on APC function. Thus, while the details of the PP2A effects on the metaphase-anaphase transition and the spindle checkpoint are still unclear, these data all indicate that Cdc55 plays a significant role in late cell cycle events. Further, the two documented cases of Cdc55-mediated PP2A effects demonstrate different modes of action of Cdc55: in the G1/S transition, Cdc55 restricts PP2A activity while in the metaphase-anaphase transition, Cdc55 serves to target PP2A activity. In all likelihood Cdc55 plays a role in a number of other processes in the cell, but even from these limited examples we can conclude that phosphatase regulatory subunits can mold catalytic activity in various ways.

The alternative PP2A regulatory subunit, *RTS1*, was isolated as multicopy suppressor of a mutated *ROX3* gene and of a *hsp60-ts* allele, highlighting an involvement of the subunit in the general stress response (Evangelista et al. 1996; Shu and Hallberg 1995). Deletion of *RTS1* results in temperature sensitivity, hypersensitivity to ethanol, and inability to utilize glycerol as a sole carbon source. At elevated temperature, *rts1Δ* cells arrest as large budded cells, with a 2N content and a non-divided nucleus. The arrest can be suppressed by overexpression of Clb2 cyclins (Zhao et al. 1997; Shu et al. 1997). Obviously, the B and the B' regulatory subunits confer different substrate specificity with little or no overlap in the function of the corresponding phosphatase complexes.

The PP2A A subunit, which is encoded by the *TPD3* gene in yeast, provides a scaffold for interaction between the catalytic subunit and the B type regulatory subunits. *TPD3* was originally isolated as a regulator

of tRNA synthesis and its deletion confers a growth defect at both low and high temperatures (van Zyl et al. 1989). The A subunit consists of 15 nonidentical repeats that fold into an elongated structure, with the C-subunit binding to the last five repeats and the B subunit binding to the first ten (Groves et al., 1999). Consistent with biochemical studies indicating that the B subunits can interact with the catalytic subunits only on the A subunit scaffold, the mutant phenotype of *tpd3Δ* is essentially the sum of the phenotypes of deletions of the two B-type regulatory subunits. However, the phenotypes resulting from loss of function of the catalytic subunits do not equal those resulting from loss of the regulatory subunits. This lack of correspondence likely reflects the fact that the regulatory subunits play both inhibitory and stimulatory roles in PP2A activity, as suggested by the studies on Cdc55, and that PP2A C participates in cellular processes through mechanisms independent of the above regulatory subunits, consistent with studies on Tap42 described below.

## 2.2

### Sit4, a PP2A Homologue

The other type 2A related phosphatase that participates in the TOR signaling pathway contains the Pph21/22 homologue Sit4 as the catalytic subunit. Strains carrying *sit4-102*, a temperature sensitive allele of *SIT4*, arrest in the G<sub>1</sub> phase of the cell cycle at restrictive temperature (Sutton et al. 1991), thus resembling the terminal phenotype of cells treated with rapamycin. Investigations of *sit4* mutant phenotypes have been complicated due to differences in the yeast strain backgrounds. These differences are caused by the presence of different alleles of the polymorphic *SSD1* gene locus (Sutton et al. 1991), which encodes an RNA-binding protein, likely involved in promoting translation of specific subsets of mRNAs (Uesono et al. 1997). In strain backgrounds with the recessive *ssd1-d* allele, *sit4Δ* is lethal and *sit4-102* confers temperature sensitivity, while a dominant *SSD1-v* allele enables a *sit4Δ* strain to grow slowly and eliminates the temperature sensitive phenotype of the *sit4-102* allele. In *sit4-102 ssd1-d* mutants Swi4 dependent transcription of G<sub>1</sub> cyclin encoding genes, such as *CLN1*, *CLN2* and *PCL1*, is reduced (Fernandez-Sarabia et al. 1992). Overexpression of either of these G<sub>1</sub> cyclins can suppress the temperature sensitivity of *sit4-102 ssd1-d* strains (Fernandez-Sarabia et al. 1992; Di Como and Arndt 1996). Besides its function

in G<sub>1</sub>/S phase progression, Sit4 regulates bud formation (Fernandez-Sarabia et al. 1992), ion homeostasis and intracellular pH (Masuda et al. 2000), amino acid permease stability, and activation of the nitrogen discrimination pathway. The latter two effects will be discussed below in more detail.

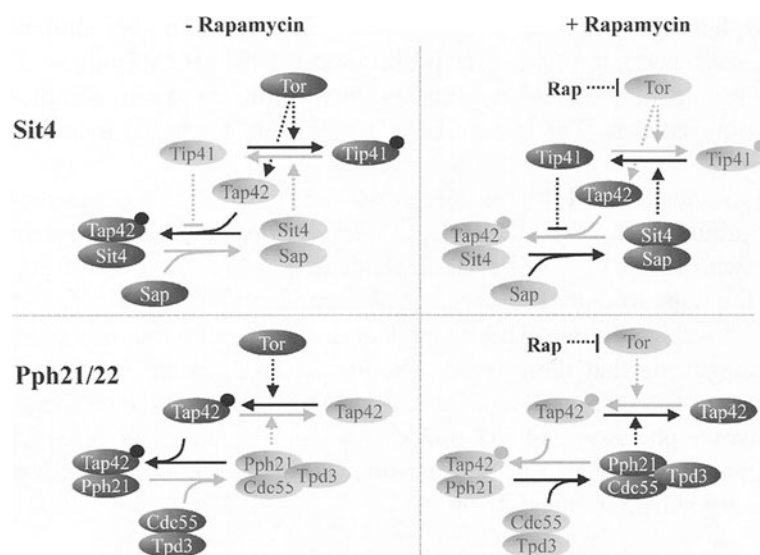
Sit4 is associated with high-molecular-weight proteins that act as regulatory subunits. Three proteins, Sap155, Sap190, and Sap185 separately interact with Sit4p in a cell-cycle-dependent manner. Deletion of any one of the Sap proteins causes no evident phenotypic difference, but strains deleted of all three exhibit a phenotype identical to that of a *sit4Δ* strain, suggesting that these three subunits act redundantly to activate Sit4 function. Overexpression of any of the Saps suppresses the temperature-sensitive phenotype of *sit4-102 ssd1-d* strain (Luke et al. 1996). A fourth gene, *SAP4*, related to the first three by sequence homology, has not yet been shown to interact with Sit4.

### 3

#### Alternative PP2A Structures—The Tap42 Connection

The realization that PP2A phosphatases are involved in the TOR signaling pathway emerged from the identification of Tap42 as a Sit4/PP2A-C interacting protein (Di Como and Arndt 1996). Tap42 forms a heterodimer with the PP2A C subunit independently and exclusively of the A and B subunits. Similarly, Tap42 binds Sit4 independently and exclusively of any of the Sap proteins. Binding of Tap42 to either PP2A-C or Sit4 depends on nutrient availability. During growth in adequate nutrients, a significant proportion of Tap42 binds to Sit4 and PP2A-C, while starvation for carbon, or particularly for nitrogen sources, results in dissolution of both complexes (Di Como and Arndt 1996; Jiang and Broach 1999). This correlation of Tap42 complex formation with growth, in conjunction with the observation that deletion of Tap42 is lethal, has prompted speculation that Tap42 may be part of a cell growth and proliferation pathway. Indeed, addition of rapamycin mimics starvation and causes dissociation of Tap42 from the phosphatase subunits.

Biochemical studies have provided direct evidence for the involvement of Tap42 in the TOR pathway. Tap42 is a phosphoprotein, whose phosphorylation state is dependent on nutrient availability (Jiang and Broach 1999). Starvation or addition of rapamycin causes dephosphorylation of Tap42. In vivo labeling experiments demonstrated that phos-



**Fig. 2** Positive feedback promotes rapid dissociation of Tap42 complexes to yield free Tap42. Diagrammed are proposed regulatory loops controlling Tor-promoted formation of Tap42-Sit4 and Tap42-PP2A-C complexes. In the absence of rapamycin (*left*) the system favors formation of the complexes as a result of Tor activation of Tap42 and inhibition of Tip41. Reduced Sit4-Sap and PP2A activities contribute to persistence of the Tap42 complexes. In the presence of rapamycin (*right*) the system favors dissolution of the Tap42 complexes and formation of Sit4-Sap, PP2A, and free Tap42. The increased activity of PP2A and Sit4-Sap enhances that transition. These regulatory loops could account for the rapid effects on downstream targets of Tor activity seen following addition of rapamycin to cells. Neither the effect of Tip41 on formation of the Tap42-PP2A complex nor the effect of Tap42 phosphorylation on Sit4-Tap42 complex has been explored. The *lighter ovals* are meant to represent reduced amounts or activities of the corresponding entity under the indicated conditions. *Black dots* represent phosphorylation

phorylation of Tap42 depends on Tor protein kinases, and immunopurified Tor phosphorylates Tap42 *in vitro*. Thus, Tap42 is a direct target of Tor phosphorylation. Dephosphorylation of Tap42 depends on PP2A, revealing reciprocal regulation of Tap42 by Tor and PP2A (Jiang and Broach 1999; Fig. 2). Interestingly, deletion of *CDC55* or *TPD3* confers rapamycin resistance (Jiang and Broach 1999), supporting the idea of competitive binding of Tap42 and the regulatory A-/B-subunits. Overexpression of Tap42 further enhances rapamycin resistance in *cdc55Δ* or *tpd3Δ* strains. However, Tap42 overexpression alone does not result in

significant enhanced rapamycin resistance, ruling out a simple regulatory mechanism in which availability of subunits determines the association. In addition, the amounts of Tap42 in yeast cells are about fivefold lower than the amounts of Pph21 and Pph22, and only a small portion of cellular Sit4 and Pph21/Pph22 interacts with Tap42. The same is true for the amount of Tap42 found in a complex with either of these phosphatases (Di Como and Arndt 1996).

Tap42 exhibits 24% identity to the  $\alpha 4$  protein in mammals, which was identified as Ig- $\alpha$ -associated protein in the B-cell receptor complex (Kuwahara et al. 1994). As is true of Tap42,  $\alpha 4$  interacts with protein phosphatase 2A, as well as with PP6, the mammalian homologue of Sit4 (Murata, et al. 1997; Chen et al. 1998; Nanahoshi 1998). Although the findings were contradictory as to whether the interaction can be altered by addition of rapamycin, at least the Tap42/phosphatase C-subunit complex formation itself seems to be conserved in eukaryotes.

Recent studies have added an intriguing kinetic component to the relationship between Tor and the Tap42 complexes (Fig. 2). In particular, both Tap42-Pph and Tap42-Sit4 complexes dissociate upon rapamycin treatment, and in both cases the dissociation is stimulated by an autocatalytic feedback loop. This insures rapid dissociation of the complexes and liberation of free Tap42 in response to loss of Tor kinase activity. Tap42-C complex formation is stimulated by Tor-dependent phosphorylation, and dissociation of this complex is stimulated by dephosphorylation catalyzed by the PP2A (A-B-C) heterotrimer (Jiang and Broach 1999). Thus, as Tor is inactivated, the increased production of PP2A heterotrimer from decay of Tap42-C heterodimers catalyzes further decay of the heterodimer by dephosphorylation of Tap42. As noted above, the rapamycin-resistant phenotype of *cdc55* or *tpd3* mutants lends credence to the postulated antagonism between the Tap42-C complex and the PP2A A-B-C complex. In a slightly more complex network that incorporates a recently identified protein Tip41, a similar feedback loop accelerates decay of Sit4-Tap42 heterodimers in response to loss of Tor activity (Jacinto et al. 2001). Specifically, Tip41 is a phosphoprotein whose phosphorylation is partially dependent on Tor and whose rapamycin-induced dephosphorylation is dependent on Sit4. Tip41 in its hypophosphorylated form inhibits interaction of Tap42 with Sit4. Accordingly, Tor activity stimulates Tap42-Sit4 interaction by inactivating Tip41. On loss of Tor activity, Sit4 stimulates dephosphorylation of Tip41, which in turn stimulates dissociation of Sit4 from Tap42, allowing further dephosphoryla-

tion of Tip41, resulting in a positive feedback loop. The presence of both these autostimulatory loops suggests urgency on the cell's part to dissolve Tap42-Sit4 and Tap42-C complexes rapidly and/or to liberate free Tap42 (see below) in response to loss of Tor activity. While the cell's urgency seems clear, the rationale for that urgency remains obscure.

#### 4

### **Phosphatases Mediate Some, but Not All, Tor-Dependent Cellular Activities**

Addition of rapamycin results in numerous changes in the yeast cell. Microarray analyses of cDNA have revealed that addition of rapamycin results in repression of a large number of genes, including translation related genes such as those encoding ribosomal proteins, RNA processing factors, cytoplasmic tRNA synthetases, etc. (Hardwick et al. 1999; Cardenas et al. 1999). In addition, a number of genes are induced by rapamycin addition, including those encoding TCA cycle components, oxidative phosphorylation machinery, enzymes affecting storage of carbohydrates, and members of the nitrogen discrimination pathway (NDP) or nitrogen catabolite repression pathway. The latter group is needed for the utilization of less-favored nitrogen sources like proline or allantoin. Also, addition of rapamycin causes a number of non-transcriptional changes in the cell, including activation of autophagy (Noda and Ohsumi 1998) and restructuring of the profile of amino acid permeases in the plasma membrane (Schmidt et al. 1998; Beck et al. 1999).

How does rapamycin addition induce these changes, or reciprocally, how does Tor activity maintain the transcriptional profiles, permease patterns, and repressed autophagy seen in actively growing cells? Several studies have placed the PP2A phosphatases and Tap42 immediately downstream of Tor in the signaling pathway. First, as noted above, rapamycin induces a rapid restructuring of the PP2A-C-containing complexes in the cell. Second, mutations affecting various phosphatase components—deletion of *CDC55* or *TPD3* or point mutations in *TAP42*—confer rapamycin resistance (see below). For none of these mutants, though, is the level of rapamycin resistance equivalent to that seen with certain *TOR1* or *TOR2* alleles. This suggests that while the phosphatases are important in executing Tor-induced activities in the cell, they do not mediate all the Tor effects. Finally, as noted below, direct examination of

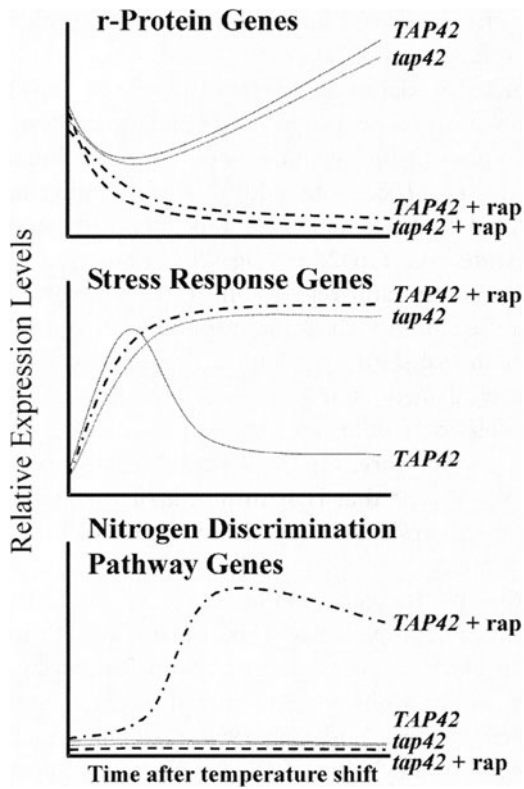


rapamycin induced events in the absence of Tap42 function has revealed a number of Tap42 dependent functions of Tor.

Most of the studies conducted to date that address the role of Tap42 in Tor-mediated or rapamycin-induced processes have relied on analysis of the *tap42-11* allele. By comparing the genome-wide transcriptional response of *TAP42* versus *tap42-11* cells to addition of rapamycin, Shamji et al. (2000) concluded that a number of rapamycin-induced transcriptional changes depended on Tap42, among them induction of NDP and TCA-cycle gene expression, and repression of genes involved in translation, including genes encoding ribosomal proteins, RNA polymerase I, rRNA processing, and translation initiation. The *tap42-11* allele causes temperature sensitive growth at 37°C, a phenotype that is fully recessive, consistent with deletion studies indicating that *TAP42* is an essential gene. At permissive temperature, *tap42-11* strains exhibit partial rapamycin resistance, a phenotype that is semi-dominant. This observation suggests that rapamycin resistance represents a gain-of-function property of the mutant protein.

NDP gene activation requires the transcription factors Gln3 and Gat1. Gat1 and Gln3 are retained in the cytoplasm by Ure2 during growth in good nitrogen sources. Upon starvation or treatment with rapamycin, both Ure2 and Gln3 become dephosphorylated, they dissociate, and Gln3 and Gat1 enter the nucleus (Beck and Hall 1999; Cardenas et al. 1999; Shamji et al. 2000). Dephosphorylation of Gln3 and/or its import into the nucleus depends on Tap42, since addition of rapamycin does not induce Gln3 import or activation of NDP gene expression in *tap42-11* cells (Beck and Hall 1999). In contrast, dephosphorylation of Ure2 appears independent of Tap42, since rapamycin-induced dephosphorylation of Ure2 occurs normally in a *tap42-11* strain (Shamji et al. 2000). Sit4 is also required for translocation of Gln3 into the nucleus following rapamycin addition.

More recent studies of the role of Tap42 on transcriptional consequences of rapamycin treatment have relied on a collection of temperature-sensitive alleles of *TAP42*. These studies have examined the effect the loss of Tap42 function has on gene expression, as well as the requirement for Tap42 function of rapamycin-induced effects (Düvel et al., 2003). The results of these experiments are summarized in Fig. 3. In the first case, the repression of ribosomal protein genes upon addition of rapamycin seems to be independent of Tap42. Not only does inactivation of Tap42 fail to cause ribosomal gene repression, but also cells can re-

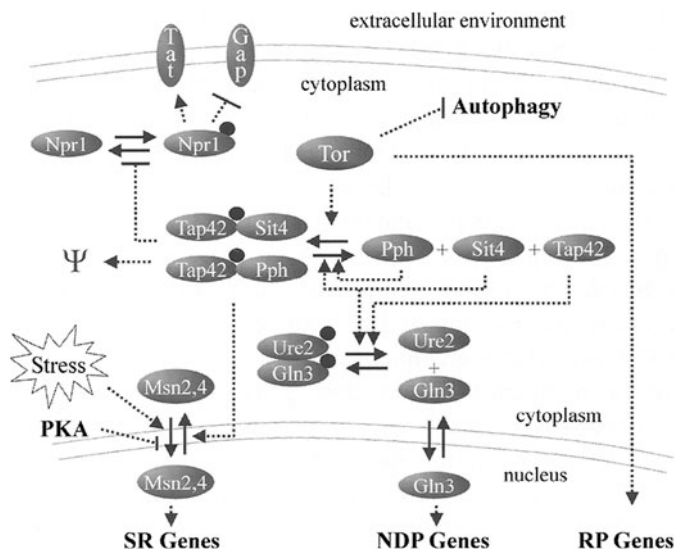


**Fig. 3** Different transcriptional effects of Tap42 inactivation: a schematic diagram of the results of transcriptional analysis of several classes of genes affected by rapamycin treatment (Düvel et al., 2003). Average expression levels of the members of each class are plotted versus time (over 1 h) following a shift from 23°C to 37°C for an untreated wild-type strain (*TAP42*), a wild-type strain treated with rapamycin at the time of the temperature shift (*TAP42 + rap*), an untreated strain carrying a *tap42* temperature-sensitive allele (*tap42*) and a *tap42* strain treated with rapamycin (*tap42 + rap*)

press ribosomal gene expression upon rapamycin treatment even in the absence of Tap42 function. In the second case, *tap42* cells exhibit a permanent activation of stress-response genes after a temperature shift. This contrasts with wild-type cells in which a temperature shift results in transient activation of stress-response genes followed by a relatively rapid return to baseline expression. These stress-response genes are under the control of the transcription factors Msn2 and Msn4. Consistent

with expression results, these factors transiently localize to the nucleus following temperature shift of *TAP42* cells, but become permanently localized to the nucleus in temperature shifted *tap42* mutant cells. Accumulation of these factors in the nucleus is not due to enhanced import following the temperature shift, but rather to inhibition of the export of Msn2/Msn4. Treatment of wild-type cells with rapamycin at the time of the temperature shift also yields permanent activation of stress response genes and trapping of Msn2/Msn4 in the nucleus. Thus, with regard to stress-response genes, loss of Tap42 function mimics rapamycin addition. In the third case, inactivation of Tap42 alone has no effect on expression of NDP genes. However, inactivation of Tap42 abolishes the ability of rapamycin treatment to induce these genes, and thus Tap42 is required for rapamycin to promote their transcriptional activation. In sum, these studies define a complex relationship between Tap42 and Tor function or rapamycin effects. For some genes, Tap42 plays no role in rapamycin-induced effects; for other genes, loss of Tap42 completely mimics rapamycin effects; and for still other genes, loss of Tap42 does not mimic but is absolutely required for rapamycin effects. These observations are discussed below.

In addition to alterations in gene expression, rapamycin treatment also causes changes in the stability of amino acid permeases and induces autophagy, a process in which cytoplasmic components are degraded as a response to starvation. Autophagy appears to be independent of Tap42, since the rapamycin resistant *tap42-11* allele does not prevent the onset of autophagy after addition of rapamycin (Kamada et al. 2000). In contrast, changes in amino acid permease stability seem to depend on Tap42. Addition of rapamycin or a shift to a poor nitrogen source causes the ubiquitination and subsequent degradation of high affinity amino acid transporters like Tat2 (Schmidt et al. 1998; Beck et al. 1999). General amino acid permeases like Gap1 are regulated in multiple ways involving transcriptional as well as posttranslational mechanisms, such as ubiquitin-dependent degradation (Grenson et al. 1983; Stanbrough and Magasanik 1995; Springael and Andre 1998). In contrast to Tat2, Gap1 is stabilized under low nitrogen conditions. Npr1, a serine/threonine kinase, acts as a key player in the inverse regulation of both Gap1 and Tat2 stability. Npr1 itself is regulated by phosphorylation that is dependent on the Tor proteins (Schmidt et al. 1998). Upon nutrient starvation or rapamycin treatment, Npr1 is rapidly dephosphorylated, implicating the activation of protein phosphatases. At nonpermissive temper-



**Fig. 4** The role of phosphatases in Tor function: proposed pathway for Tor regulation of stress-response gene expression (*SR genes*), nitrogen-discriminatory-pathway gene expression (*NDP genes*), expression of genes required for ribosome biogenesis (*RP genes*), autophagy, and *Npr1*-mediated regulation of permease levels (*Tat* tryptophan permease; *Gap* general amino acid permease). The diagram notes that translocation of *Msn2* and *Msn4* to the nucleus is stimulated by stress and inhibited by protein kinase A (*PKA*) activity. Dotted arrows indicate stimulatory effects and dotted bars indicate inhibitory effects. Black dots represent phosphorylation

ature *tap42-11* cells exhibit lower levels of *Tat2* proteins, suggesting that maintenance of *Npr1* in the phosphorylated state, i.e., inhibition of dephosphorylation, requires functional *Tap42* protein (Schmidt et al. 1998).

Recently, the involvement of the TOR pathway in pseudohyphal development has been demonstrated (Cutler et al. 2001). Yeast strains with a special pseudohyphae-promoting background change their cell morphology and budding pattern under nitrogen limitation conditions, and grow into the agar (for recent reviews see Pan et al. 2000; Gancedo 2001). Addition of low amounts of rapamycin that did not affect the vegetative growth inhibited pseudohyphal differentiation. Overexpression of *Tap42* enhanced pseudohyphal growth while *tap42-11* strains exhibited diminished pseudohyphal growth. In the case of pseudohyphae for-

mation, Sit4, but not Pph21 or Pph22, is essential for the differentiation (Cutler et al. 2001).

The negative effect of rapamycin on formation of pseudohyphae is somewhat surprising, since addition of rapamycin mimics starvation for nutrients and thus might have been expected to enhance pseudohyphal growth. More consistent with the hypothesis that rapamycin should enhance, rather than inhibit, pseudohyphal growth, earlier studies showed that deletion of *GLN3* or *NPR1* caused loss of pseudohyphal development (Lorenz and Heitman 1998). Gln3 and Npr1 are involved in the expression and stabilization, respectively, of Mep2, an ammonium permease that is necessary for pseudohyphal differentiation.

The multiple roles of phosphatases in Tor functions are summarized in Fig. 4.

## 5

### What Does Tap42 Do?

What is the actual function of Tap42 or the Tap42-phosphatase complex? Most of the biochemical studies that focused on the phosphatase activity of the complex were done with the mammalian Tap42 homologue,  $\alpha 4$ , and have led to contradictory results. The earliest study used  $^{32}\text{P}$ -labeled phosphorylase a and phosphorylated-myelin basic protein as substrates and found that the phosphatase activity of the  $\alpha 4$ /PP2A-C complex was higher than the activity of a PP2A AC dimer (Murata et al. 1997). In contrast, in vitro phosphatase assays with PP2A-C bound to  $\alpha 4$  and Tap42, respectively, using  $^{32}\text{P}$ -phosphorylated 4E-BP1 as substrate, did not reveal phosphatase activity (Nanahoshi et al. 1998). In fact, PP2A was later found to be responsible for the dephosphorylation of 4E-BP1 in mammalian cells (Peterson et al. 1999). These observations do not rule out the possibility for an active role of the  $\alpha 4$ /PP2A-C complex in targeting other substrates than PP2A. Indeed, overexpression of  $\alpha 4$  was found to enhance dephosphorylation of elongation factor-2, but did not change the phosphorylation state of p70<sup>S6</sup> kinase or 4E-BP1 (Chung et al. 1999).

The observation that several of the immediate consequences of TOR pathway inhibition involve dephosphorylation events (Npr1, Gln3, and Ure2, for examples) supports the hypothesis that Tap42 inhibits PP2A and Sit4 function. Upon rapamycin treatment Tap42 would release the phosphatase subunits and allow them to form a functional phosphatase complex. If Tap42 acts simply as an inhibitor, then loss of Tap42 func-

tion should resemble rapamycin treatment. As noted above, this prediction holds true for stress-response gene activation mediated by Msn2/Msn4. However, even in this case, the story may not be so simple. Even under rapid cell growth, which yields maximum levels of Tap42-PP2A-C and Tap42-Sit4 complexes, significant amounts of the phosphatase catalytic subunits still remain associated with their normal regulatory subunits. Thus, Tap42 could not significantly reduce the activity of phosphatase against salient substrates through a sequestration process, i.e., by simply inhibiting the phosphatase catalytic subunits to which it is bound. Rather, Tap42 might act as an antiphosphatase, either by promoting nonproductive, and thereby protective, binding to the salient substrates or by catalytic modification of the phosphatases. By whatever process, though, the genetic and biochemical analysis are consistent with Tap42 serving as a phosphatase inhibitor in at least some of the downstream functions of the TOR pathway.

For some rapamycin-sensitive processes, current observations can only be explained by invoking an active role for Tap42 in events following loss of Tor function. As noted above, inhibition of Tor function by rapamycin induces dephosphorylation and translocation of Gln3 into the nucleus, allowing activation of nitrogen-discrimination-pathway genes. Rather than mimicking the rapamycin effect, loss of Tap42 activity is required for this rapamycin-induced response. Sit4 function is also required for rapamycin induction of NDP gene expression. These observations suggest that Sit4 and Tap42 act in concert to dephosphorylate downstream targets in response to rapamycin treatment, placing Tap42 as a positive regulator of phosphatase activity. This interpretation is consistent with recent global transcriptional analysis of mutants lacking Sit4 or Pph21/22 (Düvel et al., 2003). In fact, all the results on transcriptional analysis of Tap42 can be explained by assuming that the phosphorylated form of Tap42 inhibits Sit4 and Pph21/22 activity, while the dephosphorylated form Tap42 is required for Sit4 and Pph21/22 activity.

## 6

### Future Directions

Results to date have established that phosphatases play multiple roles in Tor function and, more recently, in cellular events following loss of Tor activity. However, these results have also raised the possibility that the activities of the phosphatases in this pathway are distinct from those ex-

pected from studies on classical phosphatases. That is, Tap42 might serve not only to inhibit phosphatase activity but also to protect phosphorylated substrates from activity of other phosphatases. In addition, Tap42 appears in some cases to direct phosphatase activity against specific substrates but only under conditions in which Tap42 is not physically associated with the catalytic subunit. One possible solution to these conundrums is to propose that Tap42 covalently modifies the catalytic subunits and this modification affects the substrate specificity of the phosphatases. However appealing this explanation might be, no evidence for such a modification has yet emerged.

The most revealing experiments to clarify the role of the various phosphatase species on downstream function of Tor will be in vitro biochemical studies on the activities of these species on biologically relevant substrates. Since reconstitution experiments have not been particularly effective to date, this will require isolation of the multiple phosphatase species from native cells as well as preparation of native, phosphorylated substrates. In the interim, further transgenomic transcriptional analyses conducted with conditional alleles of phosphatase components should suggest additional involvement of phosphatases in less studied Tor processes. Finally, as with many signaling pathways, strong computational models of the signal process need to be implemented as a means of continually testing the completeness and accuracy of our representations of this critical pathway.

*Acknowledgements.* The authors would like to thank Michael Pierce and Lisa Schneper for their thoughtful comments. Work from this laboratory described in this chapter was conducted through support from the National Cancer Institute.

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# Yeast TOR Signaling: A Mechanism for Metabolic Regulation

T. Powers · I. Dilova · C.-Y. Chen · K. Wedaman

Section of Molecular and Cellular Biology, Center for Genetics and Development,  
Division of Biological Sciences, University of California Davis, Davis, CA 95616, USA  
E-mail: [tpowers@ucdavis.edu](mailto:tpowers@ucdavis.edu)

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**Abstract** Understanding how cell growth is regulated in response to environmental signals remains a challenging biological problem. Recent studies indicate the TOR (target of rapamycin) kinase acts within an intracellular regulatory network used by eukaryotic cells to regulate their growth according to nutrient availability. This network affects all aspects of gene expression, including transcription, translation, and protein stability, making TOR an excellent candidate as a global regulator of cellular activity. Here we review our recent studies of two specific transcriptional outputs controlled by TOR in the budding yeast, *S. cerevisiae*: (1) positive regulation of genes involved in ribosome biogenesis, and (2) negative regulation of genes required for de novo biosynthesis of glutamate and glutamine. These studies have raised the important issue as to how diverse nutritional cues can pass through a common signaling pathway and yet ultimately generate distinct transcriptional responses.

## 1

**Introduction**

Understanding how cell growth is regulated in response to environmental signals remains a challenging biological problem. Recent studies indicate the TOR kinase acts within an intracellular regulatory network used by eukaryotic cells to regulate their growth according to nutrient availability. This network affects all aspects of gene expression, including transcription, translation, and protein stability, making TOR an excellent candidate as a global regulator of cellular activity (Dennis et al. 1999; Rohde et al. 2001; Schmelzle and Hall 2000). Presently, we define at least three distinct areas related to TOR that remain to be elucidated. First, we wish to understand the architecture, in terms of the molecular components involved, of each of the major branches downstream of TOR. Second, we desire to understand how these individual branches interact with other, TOR-independent, cell regulatory pathways. Finally, we know very little about how TOR kinase activity is regulated, in particular with respect to upstream regulatory signal(s) that control the network. Here we review our efforts to address these issues by focusing on two specific transcriptional outputs controlled by TOR in the budding yeast, *S. cerevisiae*: (1) positive regulation of genes involved in ribosome biogenesis, and (2) negative regulation of genes required for de novo biosynthesis of glutamate and glutamine.

## 2

**TOR and Ribosome Biogenesis**

Our initial studies of TOR signaling in yeast centered on its role in the synthesis of ribosomes (Powers and Walter 1999). As the production of ribosomes represents a significant undertaking for the cell, involving well over a hundred genes and requiring a major commitment in terms of cellular resources, the decision to make new ribosomes is tightly linked with the cell's ability to sense the presence of adequate nutrients (Warner 1999). We were intrigued by a model, based on studies of mammalian cells, where TOR controls ribosome biogenesis by regulating the translation of r-protein mRNAs (Thomas and Hall 1997). This regulation involves recognition of a pyrimidine-rich sequence at the 5' termini of r-protein mRNAs, termed a 5' TOP (terminal oligopyrimidine), by the 40S ribosomal subunit (Jefferies et al. 1997; Jefferies and Thomas 1996). It

has been proposed that translation of 5' TOP mRNAs requires phosphorylation of ribosomal protein S6 by the S6 kinase (p70<sup>S6k</sup>) which, in turn, is positively regulated by TOR (Thomas and Hall 1997). Thus, in mammalian cells, TOR is postulated to regulate ribosome biogenesis by coupling growth signals to the translation of ribosomal components.

It appeared unlikely that a similar scenario would exist in yeast, based on three observations: (1) there is no identifiable homologue of p70<sup>S6k</sup> in yeast; (2) yeast mRNAs do not contain a 5' TOP; and (3) S6 phosphorylation has been shown to be dispensable for normal cell growth (Johnson and Warner 1987). Thus the question arose, what role does TOR signaling play in ribosome biogenesis in yeast? A large body of evidence has accumulated indicating that nutritional regulation of r-protein synthesis occurs primarily at the level of transcription in yeast (Warner 1999; Woolford and Warner 1991). We therefore examined whether r-protein gene expression similarly required TOR signaling. Indeed, we found that a functional TOR pathway is essential for continued transcription of r-protein genes, as well as for the synthesis and subsequent processing of 35S precursor ribosomal RNA (Powers and Walter 1999). In addition, we found that TOR activity is essential for induction of r-protein gene expression in response to improved carbon and nitrogen source availability. Finally, we observed that TOR regulates the coordinate expression of translational initiation as well as elongation factors. In an independent study, Shultz and coworkers also demonstrated that TOR regulates the expression of 35S rRNA and 5S rRNA, as well as tRNA genes (Zaragoza et al. 1998). Taken together, these results established TOR as an important regulator of ribosome biogenesis in yeast and, moreover, demonstrated that TOR activity regulates each of the three cellular RNA polymerases. A number of microarray studies have also demonstrated a role for TOR signaling in r-protein gene expression (Cardenas et al. 1999; Hardwick et al. 1999).

At present we know very little regarding the precise mechanism by which TOR controls r-protein gene expression. Inhibition of r-protein gene transcription following rapamycin treatment does not require ongoing protein synthesis, apparently excluding the requirement for de novo synthesis of a repressor protein (Powers and Walter 1999). This finding is consistent with the rapid kinetics by which r-protein transcripts disappear following inhibition of TOR activity. Indeed, effects on the synthesis of ribosomal components rank among the earliest detectable consequences of treating cells with rapamycin, arguing for a di-

rect link between TOR and ribosome biogenesis. A number of other signaling pathways also regulate r-protein gene expression, including the Ras-adenylate cyclase pathway and a specific branch of the protein kinase C (PKC) cell integrity pathway that responds to secretory defects (Warner 1999). Presently available evidence suggests that TOR acts independently of these other pathways; however, we believe that all three pathways may ultimately converge on a common set of regulators of r-protein gene expression, including the transcription factor Rap1 (Warner 1999; T. Powers, unpublished observations).

In retrospect, it appears that transcriptional control of ribosome biogenesis is likely to be a conserved activity of TOR, in that earlier studies provided evidence that RNA Pol I is inhibited by rapamycin in mammalian cells (Leicht et al. 1996; Mahajan 1994). Based on these observations, we would argue that transcriptional control of ribosomal components is likely to be a common feature of TOR signaling in all eukaryotes. By contrast, translational regulation, via S6 kinase and 5' TOP structures, may represent an additional level of control that has evolved in metazoans (Dennis et al. 1999).

### 3

#### **TOR and Nitrogen Metabolism**

In addition to ribosomes and other components involved in translation, TOR controls the expression of genes involved in a variety of nutrient-responsive cellular processes, especially those related to carbon and nitrogen metabolism (Beck and Hall 1999; Cardenas et al. 1999; Hardwick et al. 1999; Komeili et al. 2000; Shamji et al. 2000). In particular, we have demonstrated by microarray analysis that there is a significant correlation between genes regulated by the amino acid glutamine, a preferred source of assimilable nitrogen, and genes controlled by TOR, suggesting TOR may provide an important link between nitrogen metabolism and cell growth in eukaryotic cells (Komeili et al. 2000). One prominent group of glutamine-repressed genes encode permeases and enzymes involved in the uptake and catabolism, respectively, of alternative sources of nitrogen, including urea and allantoin (a product of purine catabolism). Studies by Hall and coworkers have demonstrated that many of these genes are regulated negatively by TOR, using a mechanism whereby the GATA-specific transcription factor Gln3 is sequestered in the cytoplasm (Beck and Hall 1999). This regulation also requires the cytoplas-

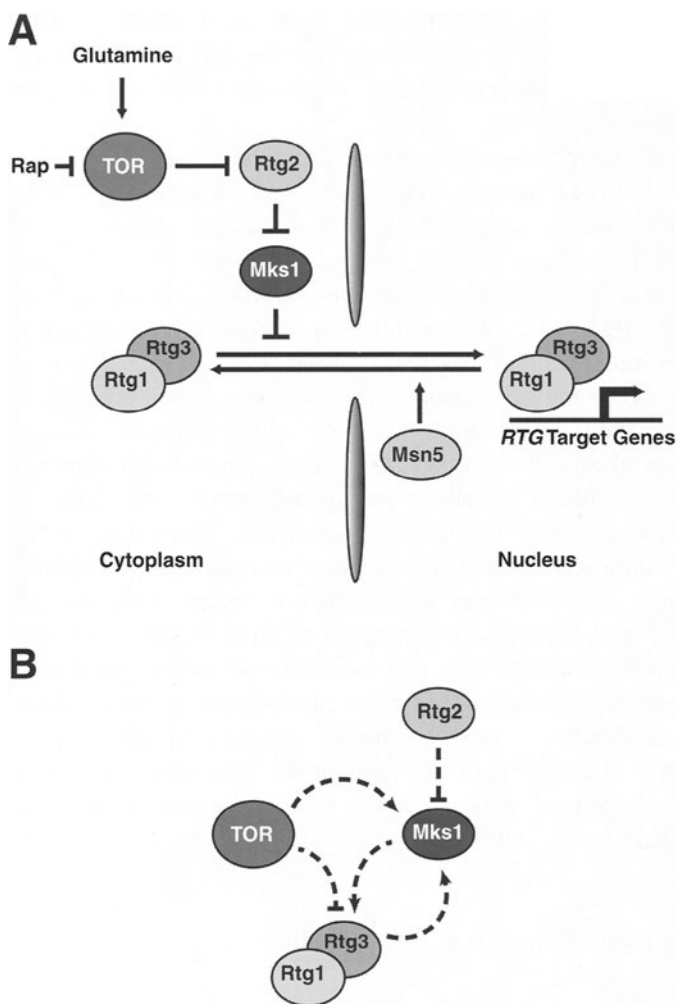
mic protein Ure2, a previously identified upstream inhibitor of Gln3 (Courchesne and Magasanik 1988), and may involve changes in the phosphorylation state of both proteins (Beck and Hall 1999; Cardenas et al. 1999; Hardwick et al. 1999).

A second prominent group of glutamine-repressed genes identified in our microarray study are termed *RTG* target genes and are required for the de novo biosynthesis of glutamate and glutamine (Komeili et al. 2000). Many of these genes include enzymes involved in the TCA and glyoxylate cycles and are regulated by the heterodimeric bHLH/Zip transcription factors Rtg1 and Rtg3 (Liu and Butow 1999). As proposed originally by Butow and coworkers (Liu and Butow 1999), one likely importance of *RTG* target gene expression is to provide adequate levels of  $\alpha$ -ketoglutarate for the production of glutamate, which is in turn required for glutamine synthesis. We observed that these genes are derepressed when rapamycin is added to cells grown in the presence of glutamine (or glutamate), demonstrating that TOR activity is required for nitrogen-mediated inhibition of these genes. The conversion of  $\alpha$ -ketoglutarate to glutamate, and ultimately glutamine, also represents a primary means by which carbon and nitrogen metabolism is linked in all cells. The finding that TOR controls this process thus underscores the importance of the pathway in regulating an essential aspect of basic cellular metabolism. Consistent with this conclusion, results of microarray analyses by Schreiber and coworkers have partitioned branches downstream of TOR into distinct gene clusters that control either carbon or nitrogen metabolism (Shamji et al. 2000).

#### 4

### Architecture of the *RTG* Branch of TOR Signaling

We were interested in understanding the mechanism by which TOR represses *RTG* target gene expression. In collaboration with Erin O'Shea's laboratory, we determined that TOR activity is required to retain the Rtg1/Rtg3 transcription factor complex in the cytoplasm when cells are grown in the presence of either glutamine or glutamate (Komeili et al. 2000). Thus, when cells grown in rich nitrogen media are treated with rapamycin, the Rtg1/Rtg3 complex moves from the cytoplasm into the nucleus, where it activates its respective target genes. We also demonstrated that rapamycin-induced nuclear entry of the complex requires Rtg2, a cytoplasmic protein previously identified by Butow and cowork-



**Fig. 1A, B** Model for how TOR and glutamine regulate *RTG* target gene expression. **A** Summary of relationships inferred from functional analyses of components involved in regulating the transcription of *RTG* target genes. When TOR is active, Mks1 is active and the heterodimeric Rtg1/Rtg3 transcription factor complex is retained in the cytoplasm. Inhibition of TOR with rapamycin or by depletion of glutamine leads to inactivation of Mks1 activity by a mechanism that requires Rtg2. Under these conditions, the Rtg1/Rtg3 complex moves into the nucleus and activates specific target genes, including *CIT2* (Liao et al. 1991). Subsequent movement of Rtg1 and Rtg3 out of the nucleus requires the activity of Msn5, a member of the importin- $\beta$  family of nuclear export factors. **B** Additional relationships between *RTG*



ers as important for activation of *RTG* target gene expression (Liao and Butow 1993). Moreover, we determined that nuclear export of the complex requires the action of Msn5, a member of the importin- $\beta$  family of nuclear export factors. Together these results extend the conclusion of Beck and Hall (Beck and Hall 1999) that an important role of TOR is to couple nutritional signals to the subcellular localization of specific transcription factors.

More recently, we have developed further our understanding of the *RTG* branch of the TOR pathway. In particular, we have determined that the likely role of Rtg2 is to inhibit the activity of another protein, Mks1, which we have shown is itself a negative regulator of *RTG* target gene activation (Dilova et al. 2002). Results of epistasis analyses indicate Mks1 acts downstream of TOR and Rtg2 as well as upstream of Rtg1/Rtg3. Consistent with a model derived from these studies (Fig. 1A), the Rtg1/Rtg3 complex enters the nucleus when the *MKS1* gene is deleted and the *RTG* target genes become expressed constitutively. This model also accounts for the observation that in *mks1* $\Delta$  cells, Rtg2 becomes dispensable for growth in the absence of glutamine (or glutamate). Moreover, it can explain why in *rtg2* $\Delta$  cells, *RTG* target genes cannot be induced by rapamycin (Dilova et al. 2002). Thus, in the absence of Rtg2, Mks1 becomes a constitutive inhibitor of the pathway.

Nevertheless, we believe that the simple, linear pathway depicted in Fig. 1A is likely to be an oversimplification of the relationships between the components involved, based on our studies of the phosphorylation states of Mks1 and Rtg3 (Dilova et al. 2002; Komeili et al. 2000). For example, we observed rapamycin-induced dephosphorylation of Mks1 that is independent of the presence of Rtg2. In addition, we also observed ra-



regulatory components, deduced from studies of the phosphorylation states of Rtg3 and Mks1 under different conditions. *Arrows* and *bars* denote increased and decreased phosphorylation, respectively, of the indicated components. In some cases, the effects of TOR appear to be antagonistic to those of other *RTG* regulatory components. (Data are from Komeili et al. 2000; Dilova et al. 2002; I. Dilova and T. Powers, unpublished observations.) Butow and coworkers have described a very similar regulatory pathway whereby the functional state of the mitochondria regulates nuclear transport of the Rtg1/Rtg3 complex in a manner also regulated by Rtg2 and Mks1 (Sekito et al. 2002; Sekito et al. 2000)

pamycin-induced hyperphosphorylation of Rtg3 that is independent of both Rtg2 as well as Mks1. These results demonstrate the existence of additional functional interactions between TOR and individual components involved in *RTG* target gene regulation (Fig. 1B).

At present we do not know how Mks1 regulates the cytoplasmic retention of the Rtg1/Rtg3 complex. Butow and coworkers have demonstrated that Rtg3 contains the nuclear localization signal (NLS) essential for import of the Rtg1 and Rtg3 complex (Sekito et al. 2000). It is therefore possible that Mks1 controls access of the nuclear import machinery to this NLS, possibly by regulating interactions between Rtg1 and Rtg3. Such a model is consistent with our results from microarray studies that indicate Mks1 is dedicated primarily to *RTG* target gene expression, at least under rich nutrient conditions (Dilova et al. 2002).

## 5

### ***RTG* Target Genes: A Convergence of Signaling Pathways**

The *RTG1-RTG3* genes were originally discovered by Butow and coworkers as required for growth when mitochondrial respiratory function is impaired, as occurs in cells that have lost their mitochondrial DNA (Liao and Butow 1993; Liao et al. 1991). This process has been termed retrograde signaling and is thought to involve signaling from the mitochondria to the nucleus (Liao and Butow 1993). The *RTG* genes have also been linked to peroxisomal function, indicating that they regulate a number of distinct metabolic functions (Chelstowska and Butow 1995; Epstein et al. 2001; Kos et al. 1995; Liu and Butow 1999; McCammon 1996). An important question raised by the studies described above is how TOR might be related to retrograde signaling. One possibility is that these constitute distinct yet overlapping regulatory pathways that converge on the *RTG*-target genes. Consistent with this idea is the close correspondence in terms of the mechanism described by Butow and coworkers for retrograde control of *RTG* target gene expression and the mechanism we have detailed for TOR-dependent control of this pathway (Sekito et al. 2002; Sekito et al. 2000).

The single exception to this overall agreement between the two responses concerns the phosphorylation state of Rtg3. Thus, while retrograde-dependent induction of the pathway involves dephosphorylation of Rtg3, we observed that rapamycin-induced activation of the pathway correlates with hyperphosphorylation of this protein (Komeili et al.

2000; Sekito et al. 2000). We have found recently that these differences may be attributable to both the strains as well as precise composition of the media used in these published studies, indicating there are yet additional levels of complexity in the regulation of this pathway (I. Dilova and T. Powers, unpublished observations).

An alternative view regarding the potential relationship between TOR and retrograde signaling is that mitochondrial function could play an indirect role, for example by providing a signal that feeds directly into TOR. In principle, this signal could be metabolic in origin, such as the intracellular concentration of glutamate or glutamine, both of which are influenced by the respiratory state of the cell (Kovacevic and McGivan 1983; Liu and Butow 1999). Indeed, in collaboration with Mike Hall's laboratory, we have recently established that glutamine acts as a signal to inhibit RTG gene expression in a manner that correlates with TOR activity (Crespo et al. 2002). Intriguingly, by contrast, we have also discovered that glutamate can repress the RTG system in the apparent absence of TOR activity (I. Dilova and T. Powers, unpublished observations). Together these results suggest that both TOR-dependent as well as TOR-independent regulatory mechanisms are likely to play important roles during RTG target gene regulation.

## 6

### **TOR: Integration of Diverse Signals**

A central question in signal transduction is how diverse upstream signals generate common transcriptional responses. An analogous question exists for TOR, namely, how do diverse nutritional signals pass through a common pathway and yet ultimately generate distinct transcriptional responses? This problem is perhaps best illuminated with a specific example involving the two branches controlled by TOR discussed above, r-protein gene expression versus *RTG* target gene expression. In the case of *RTG* target gene expression, significant expression of these genes is observed when cells are grown in media that contains ammonia or urea as nitrogen sources but not in media containing glutamine or glutamate (Komeili et al. 2000; Liu and Butow 1999). By contrast, robust expression of r-protein genes is observed under all of these conditions, demonstrating that while one branch of TOR is sensitive to the precise nitrogen source provided to the cell, another branch is not. How do we explain this differential control?

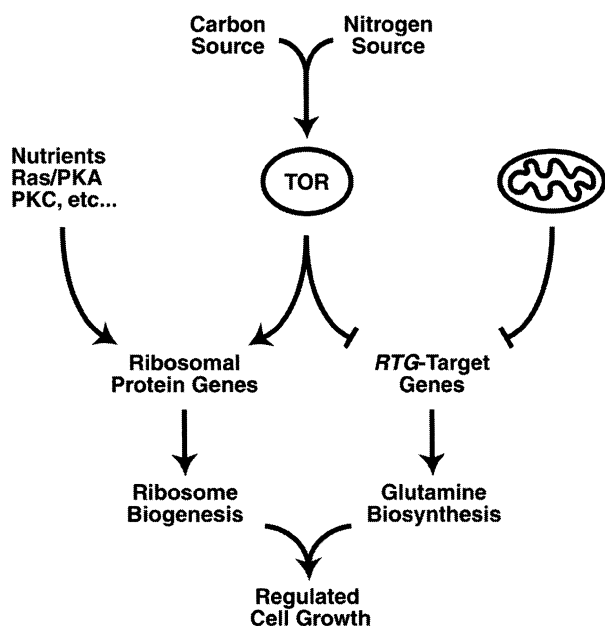


Fig. 2 Relationships between TOR signaling and other regulatory pathways that influence r-protein or *RTG* target gene expression. See text for details

According to a “smart TOR” model, TOR must be able to selectively activate or repress specific downstream target genes, depending on the precise nutritional state of the cell. Thus, for the example described above, TOR would be able to selectively repress *RTG* target gene expression when glutamine is present yet continue to activate r-protein gene expression (Fig. 2). This model is consistent with the conclusion reached by Schreiber and coworkers, based on a comprehensive set of microarray experiments, where TOR is postulated to be a “multi-channel processor” that integrates different nutritional signals (Shamji et al. 2000). This model is also consistent with the large size of the TOR kinase and the fact that it contains many potential protein-interacting domains (Schmelzle and Hall 2000). Thus, one can imagine a scenario whereby different nutritional signals would allow TOR to interact with different regulatory partners. In turn, these interactions would direct TOR kinase activity toward distinct downstream effector molecules, allowing for the differential control of gene expression described above.

An alternative view would be that the signal emanating from TOR is largely constitutive and that any observed differences in nutrient-based regulation arise from the activity of parallel pathways that act upon the same target genes. In support of this notion, regulatory pathways distinct from TOR have been proposed to control the activity of both r-protein genes as well as *RTG* target gene expression (Fig. 2). Nevertheless, it is difficult to reconcile this view with the observed dominant effects of rapamycin treatment on gene expression. Given the observed complexity in the regulation of genes controlled by TOR, it may well turn out that nutrient-based regulation requires multiple signaling pathways, including TOR. One challenge for the immediate future then is to understand how TOR activity in yeast may be modulated by changes in the nutritional environment of the cell. With the availability of powerful genetic and biochemical tools, we anticipate that yeast will continue to prove to be an important model system for furthering our understanding of this ubiquitous and essential pathway.

*Acknowledgement.* Work in the Powers' lab is supported by a Basil O'Connor Starter Research Award from the March of Dimes and by National Science Foundation Grant MCB-1031221.

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# Nutrient Signaling Through TOR Kinases Controls Gene Expression and Cellular Differentiation in Fungi

J. R. Rohde · M. E. Cardenas

Department of Molecular Genetics and Microbiology,  
Duke University Medical Center, Durham, NC 27710, USA  
E-mail: carde004@mc.duke.edu

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**Abstract** The TOR kinases were first identified in *Saccharomyces cerevisiae* as the targets of the immunosuppressive drug rapamycin. Subsequent studies employing rapamycin as a tool in yeast have given us insight into the structure and function of the TOR kinases, as well as the biological role of the TOR signaling program in transmitting nutrient signals to promote cell growth. One of the major advances from this area has been in defining an unexpected role for TOR signaling in the regulation of transcription. The identification of target genes subject to regulation by TOR has provided a platform for the dissection of the signaling events downstream of the TOR kinases. Studies aimed at understanding TOR-regulated transcription have begun to shed light on how TOR signaling cooperates with other signaling programs. In addition, the TOR pathway regulates the developmental program of pseudohyphal differen-



tiation in concert with highly conserved MAP kinase and PKA signaling programs. Remarkably, rapamycin also blocks filamentation in a number of important human and plant pathogens and the mechanism of rapamycin action is conserved in *Candida albicans* and *Cryptococcus neoformans*. The antimicrobial properties of less immunosuppressive analogs of rapamycin hold promise for the development of an effective antifungal therapy.

## 1

### **TOR Kinases in *Saccharomyces cerevisiae***

Studies in yeast were the first to identify the targets of the immunosuppressant rapamycin (Heitman et al. 1991a). The cloning and purification of FKBP12 from yeast cells anticipated that the relevant targets of rapamycin were conserved in yeast (Heitman et al. 1991b). These studies prompted a genetic screen to isolate spontaneous rapamycin-resistant mutants that revealed mutations in *FPR1* (encoding the yeast FKBP12 homolog) and two novel genes, *TOR1* and *TOR2* (for target of rapamycin; Heitman et al. 1991a). Since these initial studies, *Saccharomyces cerevisiae* has continued to lead the way in the identification and elucidation of a signal transduction pathway that responds to nutrients to control cell growth and proliferation, and has been conserved over a billion years of evolution separating yeast from humans.

The TOR kinases are proteins of approximately 280 kDa and are the founding members of a family of large proteins with a kinase domain that resembles PI-3K and PI-4K and are therefore called the PIK-related kinases. In yeast, TOR function is carried out by the products of the *TOR1* and *TOR2* genes, which share approximately 70% identity (Cafferkey et al. 1993; Kunz et al. 1993). Most of the rapamycin-sensitive functions ascribed to the TOR kinases appear to be shared between TOR1 and TOR2. However, TOR2 has been shown to have a specific rapamycin-insensitive function that regulates polarization of the actin cytoskeleton (Zheng et al. 1995; Schmidt et al. 1996). This TOR2-specific activity is essential and so while *tor1* null mutants are viable, *tor2* mutants are inviable. The yeast, TOR proteins share a conserved structure and function with the mammalian mTOR protein (also known as RAFT1, FRAP, RAPT; Cafferkey et al. 1993; Kunz et al. 1993; Brown et al. 1994; Sabatini et al. 1994; Alarcon et al. 1996a). The mTOR gene shares 39% and 43% overall identity to *TOR1* and *TOR2*, respectively. While ex-

pression of mTOR alone is unable to substitute for the function of either TOR1 or TOR2, chimeric TOR2-mTOR hybrid proteins are capable of complementing *tor2* mutants and similarly, TOR1-mTOR hybrid proteins are able to substitute for TOR1 function (Alarcon et al. 1996b). Amino acid substitutions that confer rapamycin resistance to TOR2 also confer rapamycin resistance to TOR2-mTOR hybrid proteins (Alarcon et al. 1996b). Taken together, these data demonstrate that the kinase function as well as the mechanism of rapamycin-mediated inhibition of TOR is conserved from yeast to mammals (reviewed in Cutler et al. 1999 and Rohde et al. 2001).

Rapamycin binds to the prolyl isomerase FKBP12 with high affinity and this drug-protein complex then interacts with the TOR kinases at a highly conserved FRB (FKBP12-rapamycin binding) domain distinct from the kinase domain (Chen et al. 1995; Choi et al. 1996). Complete resistance to rapamycin is achieved by mutations either in FKBP12 or in the TOR FRB domain that block the FKBP12-rapamycin TOR interaction (Heitman et al. 1991a; Cafferkey et al. 1993; Helliwell et al. 1994; Stan et al. 1994; Lorenz and Heitman 1995). How the formation of this complex results in inhibition of TOR function is not yet understood, but does not appear to involve complete inhibition of TOR kinase activity. Rapamycin treatment results in only a partial loss of kinase activity from immunoprecipitated TOR complexes (Brunn et al. 1997; Alarcon et al. 1999; Oldham et al. 2000; Peterson et al. 2000). The importance of the FRB domain is underscored by the observation that microinjection of the FRB domain into human cells results in a G<sub>1</sub> phase cell cycle arrest (Hartman et al. 2001).

In a structure-function approach, we demonstrated that partial deletion of any region of the TOR1 protein destroys the kinase activity and blocks TOR1 function (Table 1 and Alarcon et al. 1999). In addition, these studies revealed an ~500 amino acid central region of the TOR proteins that, when overexpressed, confers a dominant negative effect on cell growth and imposes a G<sub>1</sub> phase cell cycle arrest. This toxic effect could be rescued by simultaneous overexpression of *TOR1*, suggesting this domain may interact with upstream or downstream effectors of TOR. A search for such effectors demonstrated that overexpression of *TAP42*, a downstream effector of TOR, restores growth in cells overexpressing the TOR toxic domain (M.E. Cardenas, unpublished results). Similarly, overexpression of the phospholipase C gene (*PLC1*) also rescues cells from the toxic domain, suggesting that Plc1 plays a role in



viewed in Andrade et al. 2001). It has been suggested that the HEAT repeats within TOR1 mediate binding to transcription factors (Bertram et al. 2000) and localization of TOR2 to the plasma membrane (Kunz et al. 2000).

## 2

### TOR and Nutrient Signaling

Early experiments suggested a link between TOR signaling and nutrient availability (Heitman et al. 1991a). Rapamycin treatment causes cells to arrest in the G<sub>1</sub> phase of the cell cycle with dramatically enlarged vacuoles, an endpoint strikingly similar to that seen with cells that have exhausted the supply of an essential nutrient and entered a G<sub>0</sub> program (Heitman et al. 1991a; Cardenas and Heitman 1995; Barbet et al. 1996). Subsequent work strengthened the idea that the TOR kinases signal nutrient availability, and that exposure of cells to rapamycin induces many cellular processes that are triggered by nutrient depletion. These include the induction of autophagy, targeted degradation of amino acid transporters, inhibition of translation of the *CLN3* message encoding a G<sub>1</sub> cyclin, and dramatic changes in gene expression (Barbet et al. 1996; Noda and Ohsumi 1998; Schmidt et al. 1998; Cardenas et al. 1999; Hardwick et al. 1999).

Entry into G<sub>0</sub> is accompanied by hallmark changes in gene expression (reviewed in Werner-Washburne et al. 1993). These include an increase in Pol II-regulated genes required for survival in adverse conditions and a decrease in most other Pol II-regulated genes, as well as inhibition of Pol I and Pol III-regulated genes. Transcription of Pol I and Pol II messages is also defective in cells bearing mutations in protein phosphatase 2A (PP2A; van Zyl et al. 1992). Regulation of PP2A activity in response to nutrient availability is mediated in part by Tap42, a yeast homolog of the mammalian  $\alpha 4$  protein (Di Como and Arndt 1996; Jiang and Broach 1999). In response to nutrient signals that are sensed by the TOR kinases, Tap42 differentially associates with PP2A subunits or the PP2A-related phosphatase Sit4 (Di Como and Arndt 1996). These observations prompted investigation of the role of TOR signaling on Pol I and Pol III transcription (Zaragoza et al. 1998). Rapamycin was found to inhibit Pol III-directed transcription by targeting Pol III as well as the transcription initiation factor TFIIIB (Zaragoza et al. 1998). More recently, the complete sequencing of the yeast genome and the advent of genome-wide

transcription analysis allowed an intensive investigation of TOR signaling on transcription. Outstanding work in this area has produced a maelstrom of information underscoring the central role of the TOR proteins in regulating cell growth and proliferation in response to nutrients.

### 3

#### **The Identification of TOR-Regulated Genes**

The specific genes identified in genome-wide transcription array experiments have both confirmed and expanded the notion that the TOR kinases control cell growth in response to nutrients (Cardenas et al. 1999; Hardwick et al. 1999; Bertram et al. 2000; Komeili et al. 2000). The idea that the TOR kinases control cell growth in response to nutrients is reflected in the classes of genes repressed or induced upon addition of rapamycin (Cardenas et al. 1999; Hardwick et al. 1999). TOR-repressed genes/rapamycin-induced genes encode proteins required for adaptation to starvation for carbon or nitrogen and utilization of poor nitrogen sources, to carry out anapleurotic reactions induced by the retrograde response and to survive saline stress (Table 2; Cardenas et al. 1999; Hardwick et al. 1999; Komeili et al. 2000; Crespo et al. 2001). TOR-induced genes/rapamycin-repressed genes include those encoding products involved in ribosome biogenesis (Zaragoza et al. 1998; Cardenas et al. 1999; Hardwick et al. 1999; Powers and Walter 1999). In summary, the transcriptional profile of cells exposed to rapamycin underscores that the TOR signaling pathway is uniquely poised to adjust cell growth rate in response to a myriad of favorable or adverse environmental conditions.

### 4

#### **TOR-Repressed Genes**

The genes most robustly expressed in response to rapamycin treatment are those subject to nitrogen catabolite repression (NCR). During growth in the presence of favorable nitrogen sources (e.g., ammonia or glutamine) expression of the NCR genes is repressed. Upon nitrogen limitation the expression of these genes is activated to enable the cells to import and catabolize poor nitrogen sources (e.g., proline, urea, or allantoin; Table 1). Regulation of the NCR genes is complex and involves a series of feedback mechanisms as well as specific activators and repres-

**Table 2** TOR controls expression of genes subject to nitrogen catabolite repression and genes involved in ribosome biogenesis

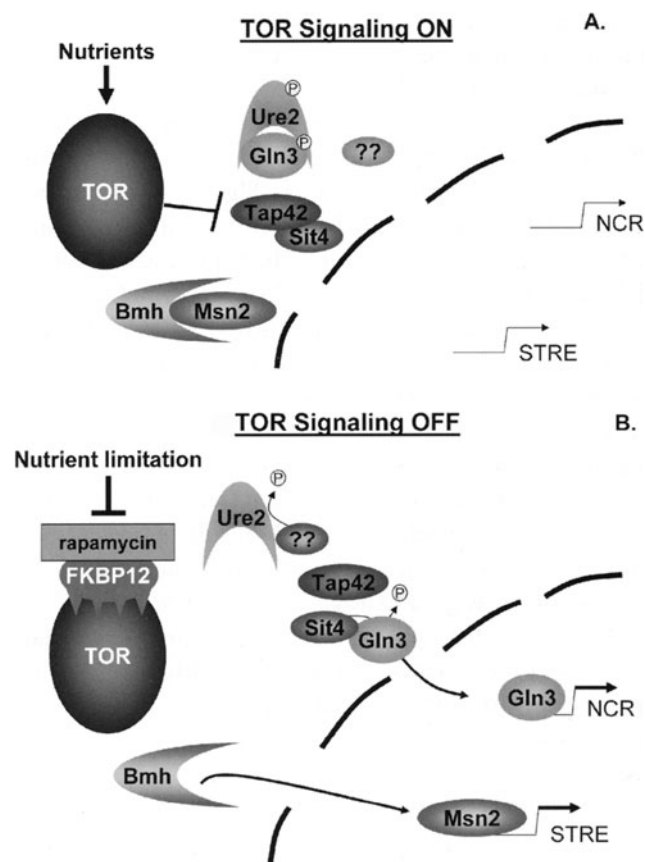
Ribosomal genes repressed by rapamycin		
Gene	Fold change	Gene product
Ribosomal proteins		
<i>RPL9A</i>	-43.2	Ribosomal protein YL11
<i>RPS26A</i>	-22.1	Ribosomal protein S26
<i>RPL6B</i>	-16.6	Ribosomal protein YL8B
<i>RPL10E</i>	-6.8	60S ribosomal protein P0
<i>CRY2</i>	-6.5	Ribosomal protein CRY1
<i>RPS28B</i>	-6.1	Ribosomal protein S18
<i>URP1</i>	-5.4	Ribosomal protein URP1
<i>RPS28B</i>	-5.2	Ribosomal protein rp28
<i>RPS5</i>	-4.6	Ribosomal protein S5
<i>RPS3</i>	-3.7	Ribosomal protein RPS3
<i>RPS26B</i>	-3.4	Ribosomal protein S26
<i>RPS28A</i>	-3.2	Ribosomal protein S28
<i>RPS25</i>	-2.5	Ribosomal protein S21
<i>RPL37A</i>	-2.4	Ribosomal protein L25
NCR genes repressed by rapamycin		
Gene	Fold change	Gene product/function
Permeases		
<i>MEP2</i>	46.2	High affinity ammonium permease
<i>DAL5</i>	19.2	Allantoate/ureidosuccinate permease
<i>GAP1</i>	7.3	General amino acid permease
<i>DAL4</i>	5.1	Allantoin permease
<i>PUT4</i>	4.5	High affinity proline permease
<i>CAN1</i>	4.3	Arginine permease
General catabolism		
<i>GDH2</i>	5.0	Glutamate dehydrogenase (NAD)
<i>GLT1</i>	3.0	Glutamate synthase
<i>GDH1</i>	1.4	Glutamate dehydrogenase (NADP)
<i>GLN1</i>	3.0	Glutamine synthetase
Specific catabolism		
<i>DUR1,2</i>	16.0	Urea degradation
<i>DAL3</i>	15.7	Allantoin utilization
<i>PUT1</i>	11.7	Proline catabolism
<i>DAL1</i>	7.9	Allantoin utilization (allantoinase)
<i>UGA1</i>	5.6	GABA catabolism
<i>PUT2</i>	2.8	Proline catabolism
<i>DAL7</i>	1.6	Allantoin degradation (malate synthetase)
<i>DAL2</i>	1.5	Allantoin utilization (allantoicase)
Transcriptional regulation		
<i>DAL80/UGA43</i>	8.2	Transcriptional repressor
<i>GAT1/NIL1</i>	3.1	Transcriptional activator
<i>DAL82</i>	2.5	Transcriptional activator

sors for certain nitrogen sources. The NCR genes subject to TOR regulation are controlled by the GATA family transcription factors Gat1 and Gln3 (Stanbrough et al. 1995; Beck and Hall 1999; Cardenas et al. 1999; Hardwick et al. 1999; Bertram et al. 2000). These transcription factors are repressed by the negative regulator Ure2 (Courchesne and Magasanik 1988; Coschigano and Magasanik 1991; Xu et al. 1995; Blinder et al. 1996). Identification of these factors has provided a useful starting point to examine the molecular mechanisms by which TOR controls gene expression. Mutation of the transcription factors that control the expression of TOR-regulated genes in many cases confers partial resistance or hypersensitivity to rapamycin. Mutations in *gln3* or *gat1* for example, result in decreased expression of NCR genes and confer significant resistance to rapamycin. In contrast, *ure2* mutations cause constitutive NCR gene expression and result in dramatic hypersensitivity to rapamycin (Cardenas et al. 1999). This analysis has now been expanded on a second genome-wide scale (Chan et al. 2000). In a "chemical genomics" approach, the rapamycin resistance of thousands of viable gene disruptions was examined. This technique identified several genes that are known to play a role in the expression of TOR-regulated genes. In addition, it also identified several genes of unknown function which are now candidates for regulation by the TOR pathway.

Work from several different laboratories has suggested a mechanism by which Gln3 activity is regulated by the TOR pathway. Initial reports demonstrated that under rich nitrogen conditions both Gln3 as well as its negative regulator Ure2 were phosphorylated and formed a complex in the cytosol (Beck and Hall 1999; Cardenas et al. 1999; Hardwick et al. 1999; Bertram et al. 2000). Treatment of cells with rapamycin or nitrogen limitation results in the rapid dephosphorylation of both proteins with kinetics that mirror induction of the NCR genes. Dephosphorylated Gln3 is then rapidly imported into the nucleus, where it drives NCR gene expression (Fig. 1; Beck and Hall 1999; Bertram et al. 2000).

In a functional genomics approach to search for gene products that were likely to direct the nuclear import and export of Gln3, it was shown that the karyopherin  $\alpha$ /Srp1 and Crm1 mediate Gln3 nuclear import and export, respectively (Carvalho et al. 2001). Moreover, Srp1 has a higher affinity for the hypophosphorylated form of Gln3, suggesting a possible mechanism for the regulated nucleocytoplasmic transport of this factor.

The Gln3-Ure2 paradigm of gene control seems to be similar for other classes of TOR-repressed genes. Both the STRE genes, which are induced



**Fig. 1** **A** In the presence of TOR signaling, TOR kinase activity favors the interaction between Sit4 and its regulatory subunit Tap42. The transcription factors Gln3 and Msn2 form complexes with Ure2 and Bmh1/Bmh2, respectively that retain them into the cytoplasm and prevent activation of target genes. **B** TOR inactivation caused by either nutrient deprivation or by the presence of rapamycin causes altered activity of PP2A phosphatases, leading to the dephosphorylation of Gln3 and Ure2. Msn2 is also released from its interaction with Bmh proteins. The transactivators are translocated to the nucleus where they are capable of activating target genes

upon a variety of environmental insults, and the genes induced by the retrograde response (a program that signals mitochondrial dysfunction) share parallels with the Gln3-Ure2 system of regulation. Similar to Gln3, the transactivators for the STRE genes, a pair of closely related proteins



Msn2 and Msn4, are restrained in the cytoplasm during noninducing conditions. Rapamycin treatment, or stresses such as heat shock or carbon starvation, results in the rapid translocation of Msn2/Msn4 to the nucleus. The cytoplasmic anchor for Msn2 and Msn4 appears to be the 14-3-3 proteins, Bmh1 and Bmh2, which in this aspect serve a function analogous to Ure2 (Beck and Hall 1999, and see Fig. 1). Parallels with the Gln3 system can also be drawn with the TOR-control of Rtg1 and Rtg3, a pair of transactivators responsible for induction of genes encoding products of the citric acid cycle needed during growth on poor nitrogen sources. The genes controlled by Rtg1 and Rtg3 are induced upon rapamycin treatment (Komeili et al. 2000). Analogous to Gln3, the Rtg1 and Rtg3 proteins are restrained to the cytoplasm during noninducing conditions and are rapidly translocated to the nucleus upon shift of cells to a poor nitrogen source or by exposure to rapamycin. Powers and coworkers determined that the protein required for nuclear export of Rtg1 and Rtg3 is Msn5 and thus deletion of *MSN5* results in the nuclear accumulation of Rtg1 and Rtg3 (Komeili et al. 2000). Remarkably, however, nuclear accumulation of Rtg1 and Rtg3 in *msn5*-deleted cells did not result in the activation of the appropriate target genes. These data suggest that TOR imposes other means of control in addition to regulated nuclear import and export of transcription factors.

## 5

### TOR Signaling Through Phosphatases

Several lines of evidence suggest that the TOR kinases control transcription through regulation of downstream type 2A protein phosphatases (PP2A). In yeast cells, the catalytic subunits of PP2A are represented by Pph21, Pph22, Pph3, and the PP2A-related protein Sit4. The regulatory subunits for PP2A include Tap42, Cdc55, and Tpd3 (Jiang and Broach 1999). In addition, a family of Sit4 associated proteins (SAPS) have been identified that act positively with the Sit4 protein and may function to target Sit4 activity towards specific substrates or may be effectors of the phosphatase (Di Como and Arndt 1996; Luke et al. 1996; Jacinto et al. 2001). Nutrient availability via TOR signaling favors Tap42 association with Sit4 and a catalytic subunit of PP2A, Pph21 (Di Como and Arndt 1996). A temperature sensitive allele of *TAP42* (*tap42-11*) has been isolated that confers resistance to rapamycin, and *SIT4* overexpression confers modest resistance to rapamycin. By contrast, deletion of *sit4* renders

the cell rapamycin hypersensitive (Di Como and Arndt 1996; Cutler et al. 2001). Collectively, these results place Sit4 and Tap42 as downstream effectors in the TOR signaling pathway (Di Como and Arndt 1996).

It is important to note that in some strain backgrounds (for example, the  $\Sigma$ 1278b background commonly used for studies of pseudohyphal growth), rapamycin-induced changes in gene expression are still observed in cells harboring the *tap42-11* allele, albeit to a lesser extent than wild-type cells (Cardenas et al. 1999; M.E. Cardenas, unpublished results). Similarly, in this strain background, deletion of either *sit4* or *pph3* only partially blocked NCR gene expression (J.R. Rohde et al., manuscript in preparation). These results indicate some degree of redundancy between Sit4 and Pph3 to regulate NCR gene expression. However, in the JK93d strain background, the rapid dephosphorylation of Gln3 observed upon rapamycin treatment was shown to be dependent on Sit4 activity, and Gln3 failed to translocate to the nucleus upon rapamycin treatment in *sit4* or *tap42-11* cells (Beck and Hall 1999). Although these results suggest that Sit4 activity controls the subcellular localization of Gln3, other PP2A catalytic subunits participate. Recently, a novel Tap42-interacting protein, Tip41, has been identified (Jacinto et al. 2001). Tip41 appears to be a negative regulator of the TOR signaling pathway. *tip41* mutations confer partial rapamycin resistance and prevent Sit4 from disassociating from its presumed negative regulator Tap42. Consistent with a role for Tip41 as a negative regulator, Gln3 fails to translocate to the nucleus upon rapamycin treatment in *tip41* cells. Taken together, these data supports a model by which nutrients signal to TOR kinases to favor a Tap42-Sit4 complex that promotes formation of a Gln3-Ure2 hyperphosphorylated complex. At this time the identity of the kinase(s) that act on Gln3 and Ure2 remains unknown and it is also not known if Sit4 is the phosphatase responsible for dephosphorylating Ure2 as well as Gln3.

## 6

### Convergence of Signaling Programs on TOR-Regulated Transcription Factors

The results from genome-wide expression studies indicate that TOR regulates a diverse set of genes (Cardenas et al. 1999; Hardwick et al. 1999). Thus, TOR signaling controls the expression of genes known to respond to other signal transduction cascades (for example, STRE-regulated

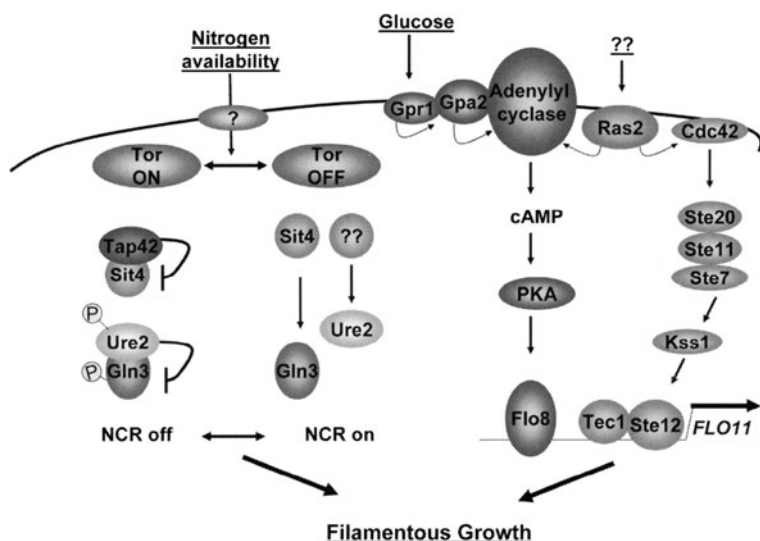
genes that are activated by specific stresses). More detailed studies indicate that TOR signaling converges with other signaling programs to regulate the activity of Gln3 and Msn2. Recently, it was shown that carbon starvation also triggers the nuclear translocation of Gln3 and the expression of NCR genes (Bertram et al. 2002). In contrast to rapamycin treatment or nitrogen limitation, however, carbon starvation causes an increase in Gln3 phosphorylation. This suggests that two distinct signaling programs act on the same transcription factor to modulate gene expression. The well-characterized Snf1 pathway was found to be responsible for these carbon starvation mediated effects on Gln3. In a similar effect, TOR inhibition with rapamycin induces the nuclear translocation of Msn2 (Beck and Hall 1999). Interestingly, the mechanisms employed by TOR and the cAMP pathway to prevent nuclear import of Msn2 appear to be distinct (Gorner et al. 2002). The regulation of Gln3 and Msn2 activity may explain how TOR signaling can cooperate with other signaling programs to direct the proper transcriptional response to distinct nutritional challenges.

## 7

### **TOR Kinases Regulate Pseudohyphal Development**

In the absence of a good nitrogen source, diploid yeast cells adopt a program of differentiation known as pseudohyphal growth (Gimeno et al. 1992; Lengeler et al. 2000; Pan et al. 2000). During this developmental program yeast cells switch their budding pattern from bipolar to unipolar, elongate, and adhere to one another by increasing the expression of the cell surface flocculin Flo11. Together, these changes result in the growth of cells as filaments emanating away from the colony in what has been termed “foraging behavior.” Intensive efforts have been dedicated to understand pseudohyphal growth in *Saccharomyces cerevisiae* because this process depends in part on highly conserved MAP kinase and PKA signaling pathways and serves as a paradigm to understand filamentous growth in other fungi (reviewed in Pan et al. 2000). For example, virulence of several important plant and human fungal pathogens depends on filamentation, either to cause disease, as in the case of *Ustilago maydis* and *Candida albicans* (Lo et al. 1997), or in other cases, to complete the life cycle as in the pathogenic basidiomycete *Cryptococcus neoformans* (Lengeler et al. 2000).

A connection between nitrogen signaling and TOR signaling prompted us to investigate a role for TOR in pseudohyphal growth. Our studies have demonstrated that TOR signaling is required for proper pseudohyphal development (Cutler et al. 2001). Subinhibitory levels of rapamycin (10 nM) that do not significantly alter growth rate block pseudohyphal growth on low ammonium media. Rapamycin prevented filament formation and agar invasion, but did not inhibit cell elongation or the switch to unipolar budding. Furthermore, the rapamycin-imposed block in pseudohyphal growth occurs through a mechanism independent from and parallel to the well-defined MAP kinase cascade, the cAMP-PKA signaling pathway, and the Sok2 repressor. At this point, it is not yet clear if TOR signaling affects filamentation by controlling transcription, translation, posttranslational modifications, or by a combination of effects. While the general translation inhibitor cycloheximide also inhibits filamentation of yeast, the mechanism by which this occurs is distinct, as certain genes are capable of suppressing the filamentous block imposed by rapamycin but not that of cycloheximide. The effects of the TOR pathway on pseudohyphal growth are mediated through the downstream effector phosphatase Sit4. Overexpression of *TAP42* or expression of the rapamycin-resistant allele, *tap42-11*, are able to overcome the block to filamentation imposed by rapamycin. These results are consistent with a model in which TOR signaling is required for proper modulation of the NCR response during filamentous growth (Fig. 2). In support of this idea, it has been shown that one of the NCR genes, *MEP2*, which encodes for the high-affinity ammonium permease, is required for pseudohyphal growth (Lorenz and Heitman 1998). Curiously, deletion of the activator of NCR-regulated genes, *GLN3*, or of *URE2*, the repressor of these genes, prevents pseudohyphal development, supporting the notion that the NCR response needs to be both induced and repressed for optimal pseudohyphal growth (Lorenz and Heitman 1998). The simplest explanation is that pseudohyphal differentiation requires both the ability to turn TOR off and on to enable the induction of the NCR-regulated genes (TOR off) and to support protein synthesis (TOR on). Pseudohyphal development has proven to be a useful system to study the molecular mechanisms of signaling pathways as well as the interplay between different signaling programs. Future work in this area should help forge a link between TOR signaling with other signaling cascades.



**Fig. 2** Multiple signaling pathways cooperate to promote filamentous growth. In response to nutrients, the TOR signaling program acts in parallel with the cAMP-PKA and the MAP kinase cascades to coordinate pseudohyphal growth in yeast. The ability to turn on and off TOR signaling appears to be required for filamentous growth

## 8

### Rapamycin As an Antifungal Drug

Current choices for antifungal treatments are limited, often cause serious side effects, and can lead to the emergence of drug resistance. This, combined with the problems that some available compounds are fungistatic rather than fungicidal, demonstrates the need for development of new antifungal compounds (White et al. 1998). Rapamycin was initially identified as a compound with potent antifungal activity towards diverse species of fungi including *Candida albicans* and the dermatophytes *Microsporum gypseum* and *Trichophyton granulosum* (Vezina et al. 1975). Since then rapamycin has been shown to inhibit the growth of many fungi, including *Candida lusitanae*, *Cryptococcus neoformans*, *Candida stelloidea*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum*, and *Penicillium* sp. Despite displaying potent antifungal activity against many important pathogens, rapamycin suffers a drawback in that it is also a very potent immunosuppressant. Recent

work, however, suggests that less immunosuppressive derivatives of rapamycin can be developed for use as antifungal drugs (Cruz et al. 2001).

The *C. neoformans* and *C. albicans* homologs of the prolyl isomerase FKBP12, as well as the homologs of the TOR kinases, have been identified and characterized (Ferrara et al. 1992; Cruz et al. 1999, 2001). In these organisms, the antifungal activities of rapamycin are mediated through formation of conserved ternary FKBP12-rapamycin-TOR complexes (Cruz et al. 1999, 2001). Analogs of rapamycin that are less immunosuppressive in humans were tested for antifungal activity towards *C. neoformans* and *C. albicans*. Two less immunosuppressive derivatives of rapamycin retained substantial toxicity towards both pathogens. The promise of antifungal drug development based on less immunosuppressive analogs of rapamycin is bolstered by two important observations in these studies. First, rapamycin and its less immunosuppressive analogs are fungicidal towards *C. albicans* and *C. neoformans*. Second, spontaneous resistance to these drugs was rare. These results suggest that the TOR signaling cascade is an excellent target for the development of broad-spectrum antifungal drugs.

## 9

### The Future of TOR

While a number of the molecular details of signaling events in the TOR pathway are beginning to be understood, a fundamental goal at this time is to identify all of the remaining components of the TOR signaling pathway in yeast. Recent work in metazoan systems has demonstrated that TOR activity is regulated by intracellular levels of ATP, phosphatidic acid, and amino acids (Fang et al. 2001; Christie et al. 2002). In addition, TOR is known to respond to specific mitogens such as insulin and has been identified as a component of the PI-3 kinase/Akt signaling program. The identities of the upstream signaling components of TOR signaling in yeast remain elusive.

The yeast model system has proven to be crucial for the identification and study of the TOR pathway, a central and conserved signaling program which for long remained elusive. We have learned a great deal with yeast, from the basic mechanics of rapamycin-mediated inhibition to the current focus on transcription. Genome-wide approaches to the study of signal transduction are being pioneered in yeast and, as a result, yeast may continue to be a leading model for the understanding of this path-

way. Many of the components identified in yeast have mammalian homologs and this will facilitate our ability to exploit this pathway's role in disease. By the same token, identification of the differences between mammalian components and the components in yeast should provide potential for development of new antifungal drugs.

*Acknowledgements.* We would like to thank Joseph Heitman for both the discovery of the TOR kinases and for critical reading of this manuscript. This work was supported by K22 Career Development Award CA94925 from NCI, National Institutes of Health (to M. E. C.).

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# Autophagy in Yeast: A TOR-Mediated Response to Nutrient Starvation

Y. Kamada · T. Sekito · Y. Ohsumi

Department of Cell Biology, National Institute for Basic Biology,  
444-8585, Okazaki, Japan  
*E-mail: yohsumi@nibb.ac.jp*

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**Abstract** TOR plays a key role in cell growth and cell-cycle progression, but in addition recent studies have shown that TOR is also involved in the regulation of a number of molecular processes associated with nutrient deprivation, such as autophagy. In budding yeast, TOR negatively regulates activation of Apg1 protein kinase, which is essential for the induction of autophagy. This review describes recent research in this field and the mechanism by which TOR mediates induction of autophagy.

## 1 Introduction

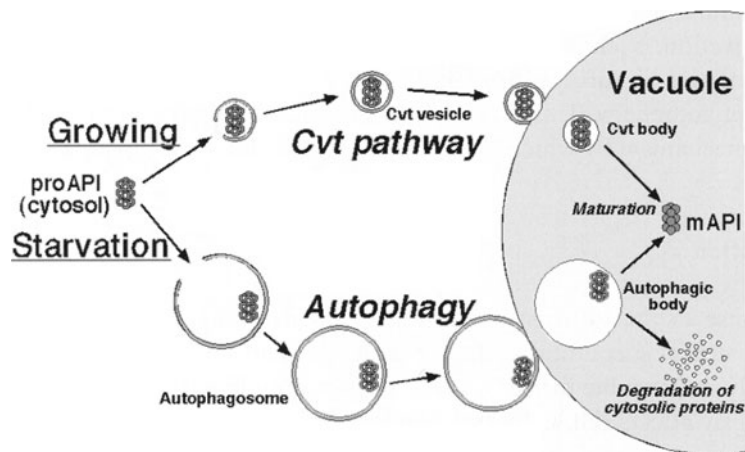
In response to the nutrient environment, cells display various phenotypes to suit new conditions. In the wild, the nutrient environment is frequently unfavorable for cells, and the adaptation to such harsh conditions largely affects cell viability. Therefore, nutrient sensing and proper responses are essential for cell life. The TOR protein is thought to play important roles in nutrient sensing. In the budding yeast *Saccharomyces cerevisiae*, rapamycin treatment, which specifically blocks TOR function,

mimics nutrient starvation (Barbet et al. 1996). It causes cells to exhibit “G<sub>0</sub>” phenotypes: cell cycle arrest at early G<sub>1</sub> (G<sub>0</sub>), reduction of protein synthesis, accumulation of glycogen, expression of a set of genes, induction of autophagy, etc. Here we review recent topics on the induction of autophagy in the yeast and discuss the involvement of the TOR signaling cascade in autophagy.

## 2

**Autophagy**

Autophagy is one of a number of responses to nutrient starvation. The bulk of cytoplasmic components are nonselectively enclosed within a double-membrane structure called an autophagosome. Then the autophagosome is transported into the vacuole/lysosome to be degraded by the resident hydrolases (Takeshige et al. 1992; Baba et al. 1994; Klionsky and Ohsumi 1999) (Fig. 1). Such turnover of bulk cytoplasm mediated by autophagy is thought to be essential for cell survival under nutrient-depleted conditions. A number of laboratories including ours have isolated genes essential for autophagy (termed *APG*, *autophagy*, or *AUT*, *autophagy*) (Tsukada and Ohsumi 1993; Thumm et al. 1994). Studies with *apg* mutants have confirmed the above hypothesis, as *apg* mutants lose via-



**Fig. 1** Switching between autophagy and the Cvt pathway in response to nutrient conditions

bility when exposed to nutrient starvation (Tsukada and Ohsumi 1993). Diploid yeast undergo meiosis and form spores when they are incubated in sporulation medium, which is deprived of a nitrogen source. Autophagy is also essential for sporulation, because *apg* diploid cells fail to form spores. Rabitsch et al. (2001) reported that *apg9* mutants were normal in meiotic nuclear divisions. Their results suggest that autophagy is required for spore formation, which is accompanied by bulk degradation of cellular proteins, but not for meiosis.

We have been investigating the function of the *APG* gene products. Genetic and morphological analyses reveal that the degradative process of autophagy shares mechanistic components with the cytoplasm to vacuole targeting (Cvt) pathway (Scott et al. 1996; Baba et al. 1997). The Cvt pathway is biosynthetic in nature, delivering resident hydrolases, aminopeptidase I (API), and  $\alpha$ -mannosidase to the vacuole (Klionsky 1998; Hutchins and Klionsky 2001). On the other hand, autophagy and the Cvt pathway differ in many aspects, including vesicle size (Baba et al. 1997). In addition, the two pathways appear to be regulated separately; the Cvt pathway is mainly utilized during growth, whereas autophagy is induced by starvation. The t-SNARE Tlg2 and Sec1-homolog Vps45 were found to be required for the Cvt pathway but dispensable for autophagy (Abeliovich et al. 1999). We showed that Sec12, an ER protein having an essential function in ER to Golgi transport, is needed for the autophagic pathway but not for the Cvt pathway (Ishihara et al. 2001). These results suggest that these two pathways are mechanistically distinct, and that switching between autophagy and the Cvt pathway should be regulated by a nutrient sensing mechanism(s) (Fig. 1).

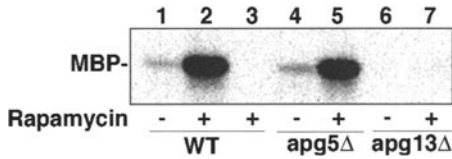
### 3

## TOR-Mediated Regulation of Autophagy

### 3.1

#### APG1-APG13

In an effort to study the mechanism of autophagy induction, Noda and Ohsumi first found that the immunophilin rapamycin, a specific inhibitor of TOR function, induced autophagy (Noda and Ohsumi 1998). This effect was abolished by the deletion of *FKB1* gene, further supporting the involvement of TOR in autophagy. This was confirmed by the observation that autophagy was also induced in a *tor2<sup>ts</sup>* mutant at nonpermis-



**Fig. 2** Apg1 is activated by rapamycin treatment. HA-tagged Apg1 was immunoprecipitated with (lanes 1, 2, 4–7) or without (lane 3) anti-HA ascite, and the resultant immunocomplex was subjected to in vitro kinase assay using [ $\gamma$ - $^{32}$ P]ATP and myelin basic protein (MBP) as a substrate. Cells were treated with (lanes 2, 3, 5, 7) or without (lanes 1, 4, 6) 0.2  $\mu$ g/ml of rapamycin for 1 h

sive temperatures. Since rapamycin treatment causes cell-cycle arrest during G<sub>0</sub>, it was possible that the drug indirectly induced autophagy via cell-cycle arrest and that cell cycle arrest at any stage could induce autophagy. This possibility was excluded, because treatment of cells with hydroxyurea and nocodazole, which arrest the cell cycle at S and G<sub>2</sub>/M, respectively, did not induce autophagy. Therefore, TOR negatively controls the induction of autophagy.

The next question that arose was how TOR regulates autophagy. The *APG1* gene encodes a protein kinase whose activity is essential for autophagy (Matsuura et al. 1997). Immunoprecipitation and in vitro kinase assays of Apg1 showed that Apg1 kinase activity was highly elevated in the cells shifted to starvation conditions (Kamada et al. 2000). Apg1 activation was also observed in rapamycin-treated cells (Fig. 2, lanes 1 and 2). These results suggest that Apg1 activation is required for the induction of autophagy and that it is mediated by TOR.

A two-hybrid screen, utilizing *APG1* as bait, led to the discovery of several Apg1-associating proteins, including *APG13* (Funakoshi et al. 1997), *APG17*, and *CVT9*. *APG17* was found to be essential for autophagy but was not required for the Cvt pathway. On the other hand, *CVT9* was required for the Cvt pathway but not for autophagy (Kim et al. 2001). The fact that Apg1 binds to proteins whose function is specific to either autophagy (Apg17) or the Cvt pathway (Cvt9) suggests that this protein complex acts as a molecular switch between autophagy and the Cvt pathway. Apg1 activation by starvation or rapamycin treatment was largely impaired in *apg13Δ* and *apg17Δ* cells (Fig. 2, lanes 6 and 7). On the other hand, deletion of *CVT9*, which is not needed for autophagy, did not affect activation of Apg1 by such treatments. The effects of deleting *APG13* and *APG17* on Apg1 activity are not due to a general autoph-

agy defect, because deletion of other *APG* genes, such as *APG5* (Mizushima et al. 1998) did not affect the activation of Apg1 (Fig. 2, lanes 4 and 5). These results indicate that the activated state of Apg1 is required for autophagy induction, and they also suggest that Apg13 and Apg17 play key role(s) in the activation of Apg1 in response to TOR inhibition. Apg13 is also found to bind to Vac8, a vacuolar protein involved in vacuole inheritance and the Cvt pathway (Scott et al. 2000).

Apg13 protein was detected by Western blot analysis as a smeared band caused by retarded migration, due to hyperphosphorylation. Hyperphosphorylation was only observed in growing cells, and Apg13 was rapidly dephosphorylated following starvation or rapamycin treatment, resulting in the disappearance of the slower migrating Apg13 forms, suggesting that phosphorylation-dephosphorylation of Apg13 is under the control of TOR.

Co-immunoprecipitation experiments revealed that TOR regulates the physical association between Apg1 and Apg13. Only a small amount of Apg13 bound to Apg1 in growing cells. In contrast, Apg1-bound Apg13 increased rapidly (in as quickly as 10 min) following rapamycin treatment. This closely paralleled the time course of Apg13 dephosphorylation. We also observed that Apg1 activation was tightly linked with Apg1-Apg13 association. These results suggested that the regulation of autophagy by TOR through Apg1 proceeds as depicted in Fig. 3. Briefly, in the cells grown in nutrient rich medium, TOR is active and causes phosphorylation of Apg13. Phosphorylated Apg13 has low affinity for Apg1 and its kinase activity remains low. In this state the Cvt pathway is activated. In contrast, in the starved (rapamycin-treated) cell, TOR becomes inactive and Apg13 is dephosphorylated. The dephosphorylated form of Apg13 tightly binds to Apg1 and confers Apg1 activation to induce autophagy.

This hypothesis was examined through experiments using an *apg13* mutant which lacks the Apg1-binding site. A truncated form of Apg13 protein, Apg13 (1–448), where Ser449 was mutated to a stop codon, has a mutation within the putative Apg1-binding site. Apg13 (1–448) does not complement the autophagic defect of *apg13Δ*, although it rescues the Cvt pathway. This result confirms the role of Apg1-Apg13 association in autophagic induction.



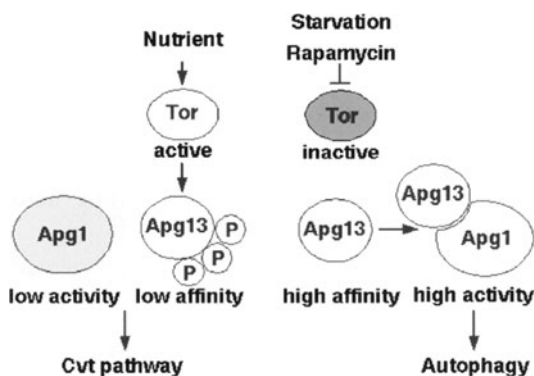


Fig. 3 Model of TOR-mediated regulation of autophagy via Apg1 complex

### 3.2

#### AUT7/APG8

Aut7/Apg8 is an essential protein for autophagy and the Cvt pathway (Lang et al. 1998; Kirisako et al. 1999). Aut7 is a ubiquitin-like protein, which is covalently conjugated to phosphatidylethanolamine (Kirisako et al. 2000; Ichimura et al. 2000). Expression of *AUT7* is increased in response to nitrogen starvation and rapamycin treatment (Kirisako et al. 1999). The deletion of any of the other *APG* genes did not affect the expression of *AUT7*, indicating that regulation of *AUT7* induction is not under control of Apg1-Apg13. Abeliovich et al. (2000) found that autophagy was attenuated by cycloheximide treatment, leading them to propose that the increase in protein levels of Aut7 (and other factors) is necessary for autophagy. We are examining whether an increase in Aut7 itself is indispensable for autophagy and whether other factors are also needed.

### 3.3

#### TAP42

It was reported that TOR signaling in yeast bifurcates into two distinct pathways (Thomas and Hall 1997). One pathway involves the small GTPase Rho1, responsible for actin organization, and is not affected by rapamycin. The other pathway involves Tap42 (Di Como and Arndt 1996), a phosphatase-associating protein necessary for the initiation of protein

translation and amino acid permease turnover, which is rapamycin-sensitive (Schmidt et al. 1998; Beck et al. 1999). *TAP42* is known to be located directly downstream of TOR, playing a requisite step in the TOR-mediated signaling, especially the rapamycin-sensitive branch (Jiang and Broach 1999). Thus, we tested whether *tap42<sup>ts</sup>* mutant (*tap42-11*) (Di Como and Arndt 1996) induces autophagy at nonpermissive temperatures (Kamada et al. 2000). This mutation also makes the Tap42 protein rapamycin-resistant at permissive temperature. We observed that accumulation of autophagic bodies in the vacuole in *tap42* cells is normal, confirming that Tap42 does not control induction of autophagy. The phosphorylation state of Apg13 is also normal in *tap42-11*; therefore, we concluded that Tap42 does not transmit a signal from TOR to Apg13.

### 3.4

#### URE2-GLN3

Induction of autophagy is regulated by nutrients. Autophagy is observed in nitrogen-, carbon-, sulfur- and phosphate-deficient cells (Takeshige et al. 1992; K. Shirahama and Y. Ohsumi, unpublished results). Commonly, nitrogen deprivation is used to study autophagy, and in this case, a nitrogen utilization mechanism might be involved in the induction of autophagy. In response to nitrogen utilization, Gln3, a transcription activator, plays an important role in expression of specific genes, such as glutamate synthase as well as ammonia and amino acid permeases (Maga-sanik 1992). Gln3 translocates to the nucleus from the cytosol and acts as a transcription factor, when cells are starved for nitrogen (Beck and Hall 1999). This translocation is controlled by its binding to Ure2 protein. TOR signaling regulates Ure2-Gln3, although the involvement of *TAP42* is controversial (Beck and Hall 1999; Cardenas et al. 1999; Bertram et al. 2000).

Chan et al. (2001) reported that expression of *APG14* is tightly regulated by TOR through Gln3. In the absence of rapamycin, the *APG14* transcript is essentially undetectable, whereas rapamycin treatment causes rapid (~10 min) and large increases in *APG14* expression. *APG14* encodes a component of phosphatidylinositol 3-kinase (Vps34) complex, which is essential for autophagy and the Cvt pathway (Kametaka et al. 1998; Kihara et al. 2001). In contrast, microarray studies revealed that rapamycin treatment only increased *APG14* by 1.4-fold (Hardwick et al. 1999). Chan et al. (2001) also showed that *ure2Δ* cells induce autophagy

in growing medium, suggesting that Ure2 is a major factor repressing autophagy under the control of TOR. However, we have found no role for the involvement of Ure2 and Gln3 in autophagy (T. Sekito and Y. Ohsumi, unpublished results). One possibility is that this discrepancy arises from the different methods employed to measure autophagy. In many cases autophagy is assayed based on microscopic visualization of the number of cells, which accumulate autophagic bodies. We usually estimate autophagy using an alkaline phosphatase (ALP) assay method (Noda et al. 1995), which in our hands is more reliable than microscopic examination.

### 3.5

#### GCN2-GCN4

Not only depletion of whole nitrogen source from the medium, but deficiency of single amino acids such as leucine and tryptophan also induce autophagy (Takeshige et al. 1992). In yeast, amino acid starvation or treatment with an amino acid analogue activates expression of genes in amino acid biosynthetic pathways (Hinnebusch 1992). This event is called “general amino acid control”, and *GCN* (general control non-derepressible) and *GCD* (general control derepressed) genes are involved in this response. Examples of GCN are Gcn2, a protein kinase which phosphorylates eIF-2 $\alpha$ , and Gcn4, a transcription factor, both of which play the key roles in this pathway. Involvement of a GCN pathway in autophagy is controversial. Natarajan et al. (2001) performed a microarray analysis following treatment of yeast with 3-aminotriazole (3AT), an analogue of histidine, and found that the increased expression of *APG1*, *APG13*, and *APG14* was Gcn4-dependent. Autophagy was also induced by 3AT treatment, but deletion of *GCN4* did not affect autophagy. In contrast, Tallozy et al. (2002) reported that in *gcn4* $\Delta$  cells, neither nitrogen starvation nor rapamycin induced autophagy, whereas in *gcn2* $\Delta$  cells rapamycin but not nitrogen starvation induced autophagy. They concluded that Gcn4 is a convergent target of regulation by the Gcn-signaling pathway and the TOR-signaling pathway.

### 3.6

#### SNF1, PHO85

Like nitrogen, the yeast cell also senses carbon and phosphate in the growing medium. As a carbon source the yeast cell prefers glucose, and when it is grown in a glucose-containing medium, expression of certain gene products becomes dispensable (e.g., proteins for galactose utilization) (Johnston and Carlson 1992). This is called glucose (catabolite) repression, and the protein kinase Snf1 acts as a key factor in glucose sensing and glucose repression.

Pho85 is a member of the cyclin-dependent kinase family (Johnston and Carlson 1992). It plays a regulatory role in expression of *PHO5*, encoding a secreted acid phosphatase. Pho5 is synthesized and secreted when phosphate levels are limiting, enabling phosphate scavenging from available organic phosphates. Pho85 binds several cyclin-like proteins, and is involved in various events such as cell-cycle control, the GCN pathway, and accumulation of glycogen.

Roach and his colleagues reported that Pho85 and Snf1 antagonistically regulate glycogen accumulation (Huang et al. 1996). They also showed that these protein kinases regulate autophagy (Wang et al. 2001); an example is deletion of the *SNF1* gene, which completely blocked autophagy induced by nitrogen starvation. They proposed that Apg1 and Apg13 are regulated by Snf1. TOR signaling controls glycogen accumulation; however, functional interaction among Snf1, Pho85, and TOR is yet to be shown.

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# The Fission Yeast TOR Proteins and the Rapamycin Response: An Unexpected Tale

R. Weisman

Department of Molecular Microbiology and Biotechnology,  
Faculty of Life Sciences, Tel-Aviv University, 69978 Tel-Aviv, Israel  
E-mail: [ronitt@post.tau.ac.il](mailto:ronitt@post.tau.ac.il)

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**Abstract** The TOR proteins are known as key regulators of cell growth in response to nutritional and mitogenic signals and as targets for the immunosuppressive and anti-cancerous drug rapamycin. The fission yeast *Schizosaccharomyces pombe* has two TOR homologues, *tor1*<sup>+</sup> and *tor2*<sup>+</sup>. Despite their structural similarity, these genes have distinct functions: *tor1*<sup>+</sup> is required under starvation, extreme temperatures, and osmotic or oxidative stress conditions, whereas *tor2*<sup>+</sup> is required under normal growth conditions. Surprisingly, rapamycin does not seem to inhibit the *S. pombe* TOR-related functions. Rapamycin specifically inhibits sexual development in *S. pombe*, and this seems to stem from direct inhibition of the *S. pombe* FKBP12 homologue. Why *S. pombe* cells are resistant to rapamycin during the growth phase is as yet unclear and awaits further analysis of the TOR-dependent signaling pathways.



## 1

**Why Study Fission Yeast?**

Yeasts are genetically tractable organisms that have long been used as model systems for studying biological processes in eukaryotes. The budding yeast *Saccharomyces cerevisiae* is the most prominent model system, and has a distinguished experimental history. It has played a pivotal role in identifying TOR as a target for rapamycin action and determining the role of FKBP12 as the mediator between rapamycin and TOR (for recent reviews, see Schmelzle and Hall 2000; Gingras et al. 2001). The use of the fission yeast *Schizosaccharomyces pombe* as a model system is comparatively recent. *S. pombe* is a unicellular ascomycete with rod-shape morphology. It grows by apical extension and divides by laying a septum in the middle of the cell. *S. pombe* is distantly related to *S. cerevisiae*, and most of the classic genetic and molecular tools of *S. cerevisiae* are also available in *S. pombe*. The genome of *S. pombe* has been completely sequenced (Wood et al. 2002), facilitating rapid genetic analysis.

The term “yeast” is somewhat misleading in relating to *S. pombe*. In phylogenetic analysis, *S. pombe* branches very early in the ascomycete lineage, before the divergence of budding yeasts and filamentous ascomycetes (such as *Neurospora* and *Aspergillus*; see Wood et al. 2002). Comparative studies of *S. pombe* and *S. cerevisiae* are especially revealing due to their evolutionary distance. If genes or regulatory strategies are conserved between these two yeasts, they are often conserved in other eukaryotes. On the other hand, if there are differences, we are directed to ask how and why these differences occur (Forsburg 1999).

Here I review our studies of TOR signaling and response to rapamycin in *S. pombe*. These appear substantially different in comparison to *S. cerevisiae*. Whereas in *S. cerevisiae* rapamycin inhibits growth and cells are arrested at the G<sub>1</sub>/G<sub>0</sub> phase of the cell cycle, in *S. pombe*, rapamycin blocks sexual development but does not inhibit growth. This observation has prompted us to investigate the cellular functions of the *S. pombe* TOR and FKBP12 homologues. I have adopted the following nomenclature to indicate whether a component is a gene or protein: *S. pombe* proteins are indicated as xyz1p and genes as xyz1, whereas *S. cerevisiae* proteins are Xyz1p and genes XYZ1.

## 2

**Rapamycin Does not Inhibit Growth in *S. pombe* but Specifically Blocks Sexual Development**

*S. pombe* multiplies primarily in the haploid state. Haploid cells have two mating types,  $h^+$  (P) and  $h^-$  (M). Upon starvation, cells commit either to sexual development or advance into stationary phase (analogous to the mammalian  $G_0$  state). Both of these responses result in cells that can survive starvation for extended periods. If a culture is composed of a single mating type, cells acquire stationary-phase physiology upon nutrient deprivation. If a culture includes cells of both mating types, a certain percentage (typically 50%–80%) of the cells conjugate (mate) to form diploid zygotes that subsequently undergo meiosis and sporulation (Davey 1998).

Our first surprise was the finding that addition of rapamycin to the growth media of *S. pombe* (up to 0.5  $\mu\text{g/ml}$ ) did not inhibit cell growth or cell division (Weisman et al. 1997). However, *S. pombe* is not indifferent to rapamycin. When we followed the effect of rapamycin (0.1–0.2  $\mu\text{g/ml}$ ) on cultures that contained both mating types, we readily noticed that rapamycin strongly inhibited cells from entering the sexual development pathway. Rapamycin inhibits sexual development at an early stage, before agglutination or conjugation. Quantitatively, the mating efficiency of cultures treated with rapamycin is reduced 9–60-fold compared to untreated cultures. Rapamycin does not affect entry into stationary phase (Weisman et al. 1997). Thus rapamycin specifically inhibits entrance into sexual development in response to starvation, but does not inhibit an alternative pathway that is activated under starvation conditions.

Why is *S. pombe* resistant to rapamycin during the growth phase? This question is intriguing given the sensitivity to rapamycin of both *S. cerevisiae* and mammalian cells. How does rapamycin cause inhibition of sexual development? To answer these questions we investigated the cellular functions of the *S. pombe* FKBP12 and TOR homologues.

## 3

**tor1p and tor2p Are Essential in Normal and Stress Conditions, Respectively**

Two TOR homologues were identified in the *S. pombe* genome and named *tor1*<sup>+</sup> and *tor2*<sup>+</sup> (Weisman and Choder 2001). The open reading

frames of *tor1*<sup>+</sup> and *tor2*<sup>+</sup> encode 2,335 and 2,337 amino acid proteins, respectively. The two *S. pombe* TOR homologues share 52% overall identity. A slightly lower level of identity, 42%–44%, is revealed when the amino acids of TOR1p and TOR2p are aligned with the human TOR protein or the *S. cerevisiae* TOR1p or TOR2p proteins. Like all other TOR proteins, the most conserved region lies in the C-terminus and contains the FRB (FKBP12-rapamycin binding) and the phosphatidylinositol 3-kinase-related domains.

Disruption analysis of *tor1*<sup>+</sup> reveals that this gene is not required under optimal growth conditions, but is critical upon nutrient depletion (Weisman and Choder 2001; Kawai et al. 2001). Cells disrupted for *tor1*<sup>+</sup> ( $\Delta$ *tor1*) exit the logarithmic phase at a lower cell density compared to wild-type cells, are abnormally long, and rapidly lose viability. The loss of viability strongly depends on the growth medium. Whereas  $\Delta$ *tor1* cells die when they reach saturation in rich medium, they maintain viability comparable to wild-type cells when grown to saturation in minimal medium (Weisman and Choder 2001). This suggests that  $\Delta$ *tor1* cells are defective in their response to a particular set of conditions. The cue involved in this putative *tor1*<sup>+</sup>-dependent signaling has not yet been determined.

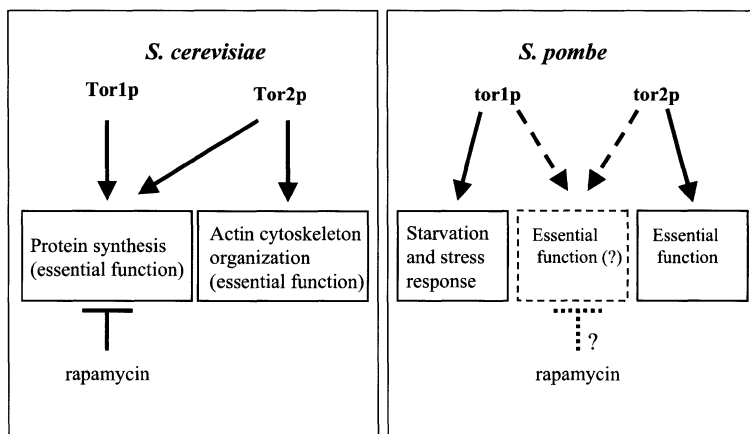
$\Delta$ *tor1* cells also exhibit a strong sterile phenotype (Weisman and Choder 2001; Kawai et al. 2001). Whereas sexual development in wild type can be induced by carbon or nitrogen starvation,  $\Delta$ *tor1* cells are unable to initiate sexual development in response to either starvation condition. Quantitatively, mating efficiency of  $\Delta$ *tor1* is reduced by several orders of magnitude compared to wild type. In summary, *tor1*<sup>+</sup> is required for the two alternative developmental responses to starvation, entrance into stationary phase, and sexual development.

*tor1*<sup>+</sup> Is also required under a wide range of stress conditions (Weisman and Choder 2001; Kawai et al. 2001).  $\Delta$ *tor1* cells are hypersensitive to extreme temperatures, high extracellular pH, osmotic stress, or oxidative stress conditions. In *S. pombe*, the stress-activated spc1p/sty1p MAP kinase cascade is important in starvation response as well as in a wide range of other stresses (Takeda et al. 1995; Kato et al. 1996; Shiozaki and Russell 1996; Samejima et al. 1998). In response to stress, spc1p/sty1p activates by means of phosphorylation the bZIP (basic leucine zipper) transcription factor atf1p that belongs to the ATF/CREB family (Takeda et al. 1995; Shiozaki and Russell 1996). This stress response mechanism is evolutionary conserved, since the mammalian *spc1*<sup>+</sup> homologues JNK/

SAPK and p38 activate the mammalian Atf1 homologue, ATF-2, in response to stress (Gupta et al. 1995). Overexpression of *atf1*<sup>+</sup> in  $\Delta$ *spc1* cells rescues the defects in stress response associated with loss of function of *spc1*<sup>+</sup> (Takeda et al. 1995; Shiozaki and Russell 1996), but does not rescue sensitivity to stress in  $\Delta$ *tor1* cells (R. Weisman, unpublished data). This finding does not support a model in which tor1p controls stress response via atf1p. Interestingly, most recently, *gad8*<sup>+</sup>, a serine/threonine kinase, was identified as a multicopy suppressor of a *tor1* mutant strain (T. Matsuo and M. Yamamoto, personal communication; Matsuo et al. 2003). Gad8p is highly similar to Ypk1p and Ypk2p in *S. cerevisiae* and belongs to the AGC kinase family that also includes the p70 S6 kinase. Genetic and biochemical analyses suggest that *gad8p* lies downstream of tor1p and is phosphorylated in a tor1p dependent manner, *gad8p* is also a substrate for phosphorylation by ksg1p, the PDK1-like fission yeast protein kinase (Matsuo et al. 2003). Thus, *gad8p* is likely to function as a common substrate for tor1p and ksg1p in a manner that resembles the regulation of mammalian p70 S6 kinase by mTOR and PDK1 (see Gingras et al. 2001).

Whereas *tor1*<sup>+</sup> is not required under normal growth conditions, disruption of the chromosomal locus of *tor2*<sup>+</sup> revealed that it is an essential gene (Weisman and Choder 2001). The cellular function of *tor2*<sup>+</sup> is currently under investigation.

The cellular roles of *tor1*<sup>+</sup> and *tor2*<sup>+</sup> are represented schematically in Fig. 1. Since in *S. pombe* rapamycin does not inhibit vegetative growth, interfere with entrance into stationary phase, or inhibit response to osmotic or oxidative stress, most of the functions of the *S. pombe* TOR proteins are resistant to rapamycin. Both rapamycin treatment and disruption of *tor1*<sup>+</sup> result in inhibition of sexual development; however, these effects appear unrelated. The inability of  $\Delta$ *tor1* cells to enter sexual development seems to be part of a general defect in responding to nutritional deprivation. In contrast, rapamycin specifically inhibits sexual development and does not interfere with other responses to starvation. Nevertheless, it is possible that rapamycin inhibits yet an unidentified function of the *S. pombe* TOR proteins (Fig. 1). Such an inhibition may explain the finding that  $\Delta$ *tor1* cells are sensitive to rapamycin under optimal growth conditions (Kawai et al. 2001; R. Weisman, unpublished data). We suggest that *tor1*<sup>+</sup> and *tor2*<sup>+</sup> may share an essential rapamycin-sensitive function. Since disruption of *tor2*<sup>+</sup> is lethal and is not rescued by overexpression of *tor1*<sup>+</sup>, we postulate that *tor2*<sup>+</sup> may exert



**Fig. 1** Schematic representation of TOR functions in *S. cerevisiae* and *S. pombe*. The illustration of the functions of *S. cerevisiae* TOR proteins is adopted from Hall (1996). The cellular functions of the *S. pombe* TOR proteins are described by Weisman and Choder (2001). *Broken lines* indicate hypothetical functions or interactions

two separate essential functions: one which is unique and rapamycin-insensitive, and another which is rapamycin-sensitive and shared with *tor1*<sup>+</sup>. This suggestion resembles the architecture of TOR functions in *S. cerevisiae* (see Fig. 1). Sensitivity to rapamycin in  $\Delta\text{tor1}$  cells could be explained if *tor1p* were more resistant to rapamycin than *tor2p*. For example, *tor1p* may bind FKBP12-rapamycin less efficiently compared to *tor2p*. Alternatively, a lower level of TOR gene expression may render cells more sensitive to rapamycin. Similarly, in *S. cerevisiae*, disruption of *TOR1* resulted in cells that are 4-fold more sensitive to rapamycin compared with wild-type cells, whereas overexpression of *TOR2* increased rapamycin resistance by 2.5–20-fold (Lorenz and Heitman 1995). It should be noted that even in the absence of *tor1p*, *S. pombe* is far less sensitive to rapamycin compared to *S. cerevisiae*; thus, further differences are likely to exist between the two organisms.

## 4

**Characterization of the FKBP12-Rapamycin Binding Domains of *tor1p* and *tor2p***

A conserved serine residue within the FKBP12-rapamycin binding (FRB) domain of TOR has been identified as the site for missense mutations conferring dominant rapamycin resistance in *S. cerevisiae* (S1972 and S1975, in *TOR1* and *TOR2*, respectively; Helliwell et al. 1994), *Cryptococcus neoformans* (S1862, Cruz et al. 1999), and human cells (S2035, Chiu et al. 1994; Chen et al. 1995). Although it is highly conserved in evolution, the cellular function of this serine is not clear. In *S. cerevisiae* or mammalian cells, mutating the conserved serine into arginine confers rapamycin resistance but does not diminish protein function. Surprisingly, mutating the equivalent serine residue of *tor1*<sup>+</sup>, S1834, into arginine results in a mutant allele that only partially complements  $\Delta$ *tor1* phenotype (Weisman and Choder 2001). Since S1834R does not affect the level of protein expression, the defective phenotype is due solely to functional differences. *tor1*<sup>S1834R</sup> did not confer rapamycin resistance in *S. pombe*, consistent with our suggestion that rapamycin does not inhibit TOR1p function in sexual development.

Interestingly, when expressed in *S. cerevisiae* under the regulation of the *S. cerevisiae* promoter *ADH1*, *tor1*<sup>S1834R</sup> but not *tor1*<sup>+</sup> conferred partial rapamycin resistance (Weisman and Choder 2001). This suggests that *tor1*<sup>S1834R</sup> can complement the function of the *S. cerevisiae* TOR proteins and that S1834 is critical for binding rapamycin in *S. cerevisiae*. Thus, despite the different roles that TOR1p and the *S. cerevisiae* TOR proteins exert in the two yeast organisms, once expressed in *S. cerevisiae*, TOR1p fulfills the role of its *S. cerevisiae* counterparts.

Our suggestion that *tor1p* can bind rapamycin-FKBP12 complexes is supported by two-hybrid assays (R. Weisman, unpublished data). The GAL4-DNA binding domain (GAL4-BD) was fused to the FRB domain of *tor1p* or *tor2p*, and the GAL4-DNA activating domain (GAL4-AD) to the *S. pombe* FKBP12 homologue. The fusion proteins were co-expressed in a rapamycin-resistant FKBP12-deficient two-hybrid host strain (a kind gift of J. Heitman, Duke University, Durham, North Carolina). The FRB domains of *tor1p* or *tor2p* were capable of interacting with FKBP12, and these interactions occurred only in the presence of rapamycin. Together, the interactions recorded by two-hybrid assays and the finding that *tor1*<sup>S1834R</sup> but not *tor1*<sup>+</sup> can suppress rapamycin sensitivity in *S. cerevisiae*

*ae* suggest that the *S. pombe* TOR proteins can bind the FKBP12-*rapamycin* complex in vivo.

## 5

### **Rapamycin Inhibits Sexual Development in *S. pombe* by Inhibiting FKBP12**

If neither Tor1 nor Tor2 is the protein target for rapamycin during sexual development, then what is the target? Analysis of the *S. pombe* FKBP12 homologue has shed light on this issue. *S. pombe* has a single FKBP12 homologue (*fkh1*<sup>+</sup>) that shows extremely high homology with the *S. cerevisiae* FKBP12 protein (overall 70% identity) and remarkable similarity with the human FKBP12 (overall 55% identity). In *S. cerevisiae*, disruption of the FKBP12 homologue (*FPR1*) results in a viable, rapamycin-resistant phenotype (Heitman et al. 1991; Koltin et al. 1991). This finding helped in establishing the current model for rapamycin action, in which FKBP12 is required to form a toxic protein-drug complex. Surprisingly, disruption of the *S. pombe* FKBP12 homologue ( $\Delta$ *fkh1*) results in a phenotype that is similar to treatment of wild-type cells with rapamycin.  $\Delta$ *fkh1* cells grow well under optimal growth conditions, are able to acquire stationary phase physiology, but cannot initiate sexual development (Weisman et al. 2001). The similarity between the phenotype of  $\Delta$ *fkh1* cells and rapamycin treatment suggests that rapamycin may exert its inhibitory effect by inhibition of fkh1p. A screen for *fkh1* mutants that confer rapamycin resistance identified five amino acids, F47, C49, L56, I92, and F100, that are critical for the effect of rapamycin in *S. pombe* (Weisman et al. 2001). Structural studies predict that the equivalent amino acids in the human FKBP12 homologue closely interact with rapamycin (Van Duyne et al. 1993). Presumably, the rapamycin-resistant *fkh1* mutants are impaired in rapamycin binding; hence they confer drug resistance. Alternatively, rapamycin may bind the mutant protein but fail to inhibit its activity.

The *S. cerevisiae* FKBP12 homologue has not been implicated in sexual development; however, introduction of *FPR1* into  $\Delta$ *fkh1* rescues its sterile phenotype. Conversely, introduction of *fkh1*<sup>+</sup> into *S. cerevisiae* cells lacking *FPR1* restores rapamycin sensitivity, suggesting that fkh1p forms a toxic complex with rapamycin that binds and inhibits the *S. cerevisiae* TOR proteins (Weisman et al. 2001).

When expressed in *S. cerevisiae* cells that lack *FPR1*, the *S. pombe fkh1* mutants exhibit different effects. The F47S mutant completely fails to restore rapamycin sensitivity, whereas F100L and C49R restore partial rapamycin sensitivity. Thus, F47, F100, and C49 also play an important role in the response to rapamycin in *S. cerevisiae* cells. Somewhat surprisingly, the mutant L56F efficiently restores rapamycin sensitivity in *S. cerevisiae*, suggesting that it can efficiently form a toxic complex with rapamycin. The difference in the effects of the L56F mutant on the response to rapamycin in the two yeasts might reflect the difference in rapamycin response. Whereas in *S. pombe* rapamycin is likely to inhibit FKBP12 activity, in *S. cerevisiae* rapamycin inhibits TOR activity in association with FKBP12.

## 6

### Summary

Each of the two *S. pombe* TOR homologues carries out a distinct function. *tor1*<sup>+</sup> is required under starvation and other stresses, and *tor2*<sup>+</sup> is required under normal growth conditions. The cellular function of *tor2*<sup>+</sup> is yet to be determined. The requirement for tor1p in response to starvation suggests that like other TOR proteins (Schmelzle and Hall 2000; Gingras et al. 2001; Rohde et al. 2001), tor1p may participate in signal transduction pathways that are dedicated to nutrient sensing. However, since tor1p is also required under a variety of stress conditions, including osmotic or oxidative stress, its role extends beyond nutrient sensing. Interestingly, a link between *S. cerevisiae* TOR and salt stress was recently suggested (Crespo et al. 2001). In addition, recent data indicate that mTOR (FRAP) is involved in sensing osmotic stress (Desai et al. 2002). Thus, TOR may play a conserved role in regulating growth in response to a wide variety of environmental changes, besides nutritional changes.

Rapamycin does not inhibit growth of *S. pombe* cells under normal growth conditions, nor does it perturb their entrance into stationary phase, or their growth under osmotic or oxidative stress conditions. The most evident effect of rapamycin in *S. pombe* is its inhibition of the sexual development pathway. This inhibitory effect is likely to be the result of a direct inhibition of the fission yeast FKBP12 homologue. Why *S. pombe* is resistant to rapamycin during the growth phase, or indeed, why most of the functions of the *S. pombe* TOR proteins are resistant to rapamycin is as yet unclear. To answer these questions we must further



clarify the cellular roles of the TOR proteins, identify their substrates, and determine the molecular pathways that underlie the specificity of tor1p and tor2p. Revealing the molecular basis for rapamycin resistance in *S. pombe* may illuminate different strategies in growth regulation and cell cycle progression in different cell types.

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# Plant Growth and the TOR Pathway

B. Menand<sup>1</sup> · C. Meyer<sup>2</sup> · C. Robaglia<sup>1</sup>

<sup>1</sup> CEA Cadarache DSV DEVM, Laboratoire du Métabolisme Carboné,  
UMR 163 CNRS CEA, Univ-Méditerranée UMR 163,  
Saint-Paul-lez-Durance, France  
*E-mail: robaglia@cea.fr*

<sup>2</sup> Unité de Nutrition Azotée des Plantes, INRA, Versailles, France

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**Abstract** In mammalian, insect, and yeast cells, TOR proteins are essential regulators of cell growth in response to environmental signals including nutrients, mitogens, and stresses. Although many aspects of the TOR-dependent signalling pathway are conserved between animals and fungi, important differences have also been found and are likely to be related to the ecophysiological adaptations of these organisms. The TOR protein also exists in plants. This review will first discuss specific aspects of plants concerning the contribution of cell growth to overall growth, as well as their responses to nutrient starvation, with emphasis on recent results obtained through genetic analysis in the model plant *Arabidopsis*

*thaliana*. This is followed by the current status of the genetic analysis of the *TOR* gene in this plant and the search for potential members of a *TOR* pathway in the *Arabidopsis* genome.

## 1

### Introduction

In mammalian, insect, and yeast cells, *TOR* proteins are essential regulators of cell growth in response to environmental signals including nutrients, mitogens, and stresses. Although many aspects of the *TOR*-dependent signalling pathway are conserved between animals and fungi, important differences have also been found and are likely to be related to the ecophysiological adaptations of these organisms. The *TOR* protein also exists in plants. This chapter will first briefly review specific aspects of plants concerning the contribution of cell growth to overall growth, as well as their responses to nutrient starvation, with emphasis on recent results obtained through genetic analysis in the model plant *Arabidopsis thaliana*. This is followed by the current status of the genetic analysis of the *TOR* gene in this plant and the search for potential members of a *TOR* pathway in the *Arabidopsis* genome.

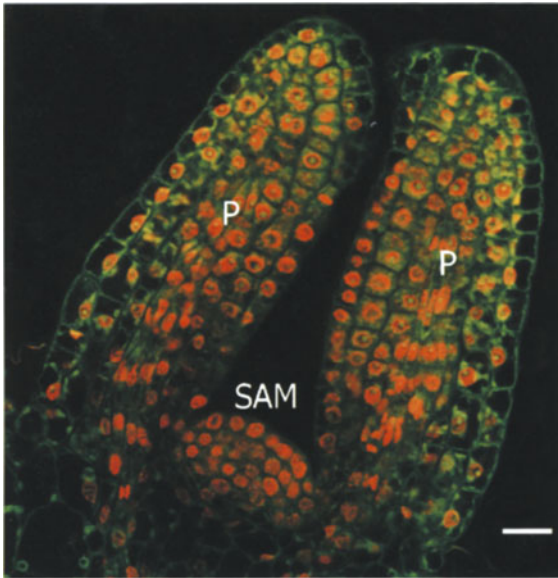
## 2

### Plant Growth

#### 2.1

##### Different Modes of Cell Growth in Plants

The size of an organism is defined by the size and number of its cells. During plant life, new cells are continually formed by specialized structures, the meristems. Shoot apical meristems (Fig. 1) form all aerial organs (leaves, stem, and flowers) and axillary meristems in an iterative fashion. The root apical meristem is at the origin of the root system, but secondary lateral root meristems do not derive directly from the primary root meristem. They are rather initiated from pericycle cells, an internal tissue of the root (Beeckman et al. 2001; see Fig. 3A). Meristems are organized in multicellular domains where cells are small, densely packed with cytoplasm, and undergo cell divisions at different rates (Fig. 1). Meristematic cells undergo cell growth and division in a coupled fashion. Both the density of their cytoplasm and the small size of the vacuole



**Fig. 1** The *Arabidopsis* shoot apical meristem (SAM). Longitudinal section into the SAM of an 8-day-old plantlet obtained by confocal microscopy. The nucleus is stained in red with propidium iodide; the cytosol is stained in green with rhodamine 123. The dark zones are vacuoles. The two first leaves primordia (P) surround the SAM. Proliferating cells with dense cytoplasm are found in the SAM. Cells undergoing expansion are found at the tip of the primordia. (From Autran and Traas 2001, copyright 2001 Médecine et Sciences Masson, France). Bar, 20  $\mu$ m

imply that growth must be sustained by an important level of protein synthesis.

Following their progressive exit from the meristematic zones and during the formation of organs, plant cells can increase their size by up to 100 times. This process, called expansion, accounts for a large part of the final plant size. Cell expansion is driven by water accumulation into a large vacuole and by the enzymatic polymerization of sugar backbones for cell wall production, a polymeric network of crystalline cellulose microfibrils embedded in a hydrophilic matrix of hemicelluloses and pectins. Several cycles of endoreduplication can also occur. This change in growth mode is accompanied by a decrease of the division potential. For example, during *Arabidopsis* leaf development, dividing cells are initially observed throughout the young leaf, but gradually become restricted to the basal portion of the blade, then to the petiole, and become almost

absent in adult leaves (Donnelly et al. 1999). However, asymmetric divisions of epidermal cells associated with stomatal development occur beyond this zone of cell proliferation. In the root, pericycle cells keep some potential for division to initiate secondary root meristems (Beeckman et al. 2001). Compared to animals, plants can therefore reach large size with a comparatively small number of cells. The plant cell expansion can be viewed as an adaptation to increase in size economically because plants, as sessile organisms, had to optimize their capacity to explore their environment to collect essential resources such light, mineral nutrients, and water (Cosgrove 1997).

In specialized organs, other types of growth can be observed. The endosperm of angiosperms is a triploid tissue, which develops during embryogenesis by undergoing several cycles of nuclear division without cell division. This results in the formation of a syncytium containing up to 200 nuclei before cellularization occurs. Each syncytial nucleus is surrounded by a dense cytoplasm (Mansfield and Briarty 1990).

In contrast, pollen tube and root hairs grow in a polarized manner with a large vacuole occupying most of the cellular space. The cytoplasm is reduced and accumulates at the apex of the cell. The growth of pollen tube and root hairs involves the trafficking of membrane vesicle to transport cell-wall precursor and an intense reorganization of the cytoskeleton (Hepler et al. 2001).

## 2.2

### Coordination Between Cell Growth and Cell Division in Plants

During the formation of organs, the initially small meristematic cells progressively increase in size by expansion while still dividing. Inhibition of plant cell division can be compensated by an increase in cell growth. This was first demonstrated by the continuous growth of gamma ray-irradiated wheat foliar primordia (Haber 1962). More recently, Hemerly et al. (1995) reported that the overexpression of a dominant negative CDK leads to plants with normal morphology and a reduced number of larger cells. The expression of KRP2, an inhibitor of CDK, also induces cell enlargement while altering leaf morphology, but the overall patterning of the plant is maintained (De Veylder et al. 2001b). These observations suggest a supracellular control of growth. Land plants are phylogenetically related to syncytial algae, in which growth and morphogenesis are independent of cell division. Indeed, the cells of

multicellular plants are still forming a cytoplasmic continuum connected by intercellular structures named plasmodesmata. The organismal theory of plant development postulates, in opposition to the cell theory, that cell division does not drive plant growth and morphogenesis and that it is subordinated to growth control; "this is the plant that makes the cells and not the cells which make the plant" (Kaplan and Hagemann 1991). On the other hand, modifying the expression of plant cell-cycle genes, closely related to animal cell-cycle genes, can modify growth rates, showing that cell division is nevertheless an important component of growth (reviewed in Meijer and Murray 2001). For example, overexpression of *Arabidopsis* cyclin D2 in tobacco increases the growth rate by reducing the time spent in G<sub>1</sub>, without modifying meristematic and post-mitotic cell size (Cockcroft et al. 2000). The overexpression of CKS1At, a CDK-associated protein, leads to an increase of the length of G<sub>1</sub> and G<sub>2</sub> phases, a diminution of meristem size, and a reduction of growth rate (De Veylder et al. 2001b). In plants, the possibility that D-type cyclins can be direct growth promoters, instead of G<sub>1</sub>/S regulators, as is the *Drosophila* cyclin D-CDK4 complex, has not yet been investigated (Datar et al. 2000).

During the formation of organs, as in foliar primordia, plant cells can undergo simultaneously premitotic growth and divisions and growth by expansion. The *aintegumenta* gene has been identified as a gene controlling the growth and final number of cells in a fully grown organ (Mizukami and Fischer 2000). However, the control of the size at which meristematic cells divide may influence the number of divisions that they will ultimately undergo. The factors governing both the size reached by postmitotic cells and the final organ size are largely unknown and will certainly prove to be an interesting area of future investigations. An interesting example of the close relationship between expansion and cell division is that the proliferation of cells leading to the formation of a foliar primordium, on the side of the shoot apical meristem, can be triggered by the local application of agents that stimulate the loosening of the cell wall, such as the protein expansin and the phytohormone auxin (Pien et al. 2001; Reinhardt et al. 2000). This leads to the idea that meristematic cell divisions are somehow constrained by the mechanical pressure exerted by the cell wall.

### 2.3

#### Cell Growth Control by Phytohormones

Plant hormones are small, mostly organic, molecules involved in signalization between cells. The addition of exogenous hormones and the analysis of mutants affected in their synthesis or perception show that some of them are involved in the regulation of cell proliferation and cell expansion (Kende et Zeevaart 1997). Gibberellins induce cell elongation in the aerial part, and mutants unable to produce or perceive them are dwarfed (Richards et al. 2001). Auxins, derived from tryptophan, and cytokinins, derived from purines, were discovered due to their capacity to synergistically promote proliferation of cells and tissues in vitro. Their relative concentrations affect cell differentiation: a high auxin/cytokinin ratio promotes rhizogenesis, whereas the reverse allows the formation of buds from undifferentiated callus. Auxins are known to stimulate cell elongation, and their perception involves the auxin binding protein, ABP1. Inactivation of the single *Arabidopsis* *ABP1* gene leads to a lethal embryonic phenotype where cells of the embryo still divide, whereas pattern acquisition by elongation of specific cells does not occur (Chen et al. 2001). This, together with the down-regulation of the *ABP1* gene in tobacco cell cultures, suggests that auxins are involved in the coupling between cell expansion and division but not in the cytosolic mass increase associated with proliferation. On the other hand, a substantial body of evidence links cytokinins to cell division (D'Agostino and Kieber 1999). Recent experiments show that overexpression of the G<sub>1</sub> cyclin D3 allows callus proliferation in the absence of cytokinins (Riou-Khamlichi et al. 1999). It seems that cytokinin signalling involves a two-component system, initiated by membrane-bound histidine kinases, which perceives the hormone and is relayed by histidine phosphotransmitters (Hwang and Sheen, 2001). The precise mode of action of auxin and cytokinins on their multiple targets remains a matter of debate.

### 2.4

#### Adaptative Morphogenesis in Response to Nutrients

The development of plant organs, including lateral roots, does not follow a rigid predetermined plan but is mainly controlled by environmental signals. Indeed, plants need to continuously adjust their growth pattern



to their environment; for instance, as they are immobile organisms, they must direct and expand their roots towards nutrient-rich zones. The development of lateral roots is usually the major way used by plants to explore and forage nutrient patches in soil. Lateral roots are formed by G2-blocked pericycle cells that resume cell cycling (Beeckman et al. 2001). This primordium forms a new meristematic region which can be activated or remain quiescent. This activation seems to be mainly triggered by the sensing of nutrients (N and P being the most effective) in the root vicinity (Forde and Lorenzo 2001). It has thus been shown that in *Arabidopsis*, as in other plant species, a localized supply of nitrate re-activates meristematic cell proliferation and root elongation (Zhang and Forde 1998; Forde and Lorenzo 2001). This induction of root development is also modulated by the internal nutrient status of the plant. Plants starved for nitrogen respond more strongly to external signals than nitrogen-replete ones (Forde and Lorenzo 2001). Interestingly, nitrate stimulates the activation of lateral root meristems but hardly stimulates the lateral root initiation (i.e. the formation of the meristematic region). Little is known about the transduction of this signal or of the genes involved in this process. Auxin plays an important role in lateral root initiation, and auxin-resistant mutants show a decreased degree of lateral root initiation (Malamy and Benfey 1997; Zhang et al. 1999). Genes involved in nutrient sensing are as yet unknown or controversial in higher plants, but a transcription factor from the MADS-box family (ANR1) has been shown to be required for nitrate stimulation of lateral root elongation (Zhang and Forde 1998).

Although plants are carbon autotrophs, certain non-photosynthetic organs and cells (sink tissues), notably roots, meristems, and seeds, can be considered carbon heterotrophs. During senescence, nutrients stocked in adult leaves are reallocated to reproductive organs, following organized degradation of cell components to provide nutrient reserves for the next generation; this process is antagonized by cytokinins (Gan and Amasino 1995). Studies of the response of plant cells to carbon starvation have been performed with excised root tips and cell cultures (reviewed in Yu 1999). Plant cells respond to sucrose starvation by activating the use of alternative carbon sources (lipids, starch, proteins), and by repressing cellular functions that use sugars (starch synthesis, protein synthesis and storage, cell cycle, nitrate assimilation, respiration, and sucrose metabolism). This control of cell activity is associated with an increase of vacuole size and an important autophagic activity (Aubert et

al. 1996). Similar results have been observed with whole plants, as proteolysis is rapidly induced, first in sink tissue, when maize plants are submitted to extended darkness (Brouquisse et al. 1998). Riou-Khamlichi et al. (2000) observed that the level of cyclin D2 and D3 mRNAs, and also the activity of the corresponding CDKs, strongly increase when sucrose is added to starved *Arabidopsis* cells. This transcriptional control is not a consequence of the cell-cycle activation, since it is not inhibited by the protein synthesis and cell-cycle inhibitor cycloheximide. Thus, the sugar availability could be sensed at the level of the cell cycle by a signalling pathway involving D cyclins. In addition, elevated levels of exogenous CO<sub>2</sub>, which is converted by the photosynthetic process into sugars, increase epidermal cell elongation and division rate (Masle 2000).

### 3

## Presence of a TOR Pathway in Plants

### 3.1

#### Resistance of Plants to Rapamycin

Rapamycin inhibits TOR proteins in *Saccharomyces cerevisiae* and animals, by forming a bridge between the FKBP-rapamycin binding domain (FRB) of TOR and FKBP12. This inhibition of TOR activity is responsible for the antiproliferative effect of this antibiotic against organisms as different as budding yeast, fungi, *Drosophila*, and mammals. However, the vegetative growth of land plants seems to be unaffected by rapamycin (Menand et al. 2002). This resistance could have appeared during green algae evolution, since this phylum contains both rapamycin-sensitive (*Chlamydomonas reinhardtii*) and rapamycin-resistant (*Protheca segbwema*) species (Menand et al. 2002; Baker et al. 1978). The resistance of plants to rapamycin is not the consequence of the absence of a TOR pathway. Indeed, a plant TOR (*AtTOR*) gene, essential for early development, has been identified and characterized in *Arabidopsis* (Menand et al. 2002). The AtTOR FRB domain is able to interact, in a rapamycin-dependent manner, with the *S. cerevisiae* FKBP12, but not with AtFKBP12, suggesting that AtFKBP12 is responsible for the resistance to rapamycin (Menand et al. 2002; B. Menand and C. Robaglia, unpublished data). However, the possibility cannot be excluded that rapamycin cannot enter plant cells or that a detoxication pathway exists in plants. Furthermore, it has been suggested that in *S. pombe*, SpTORs and SpFKBP12

proteins can interact with rapamycin without inhibiting TOR activity (Weisman et al. 2001). This could be also the case in plants.

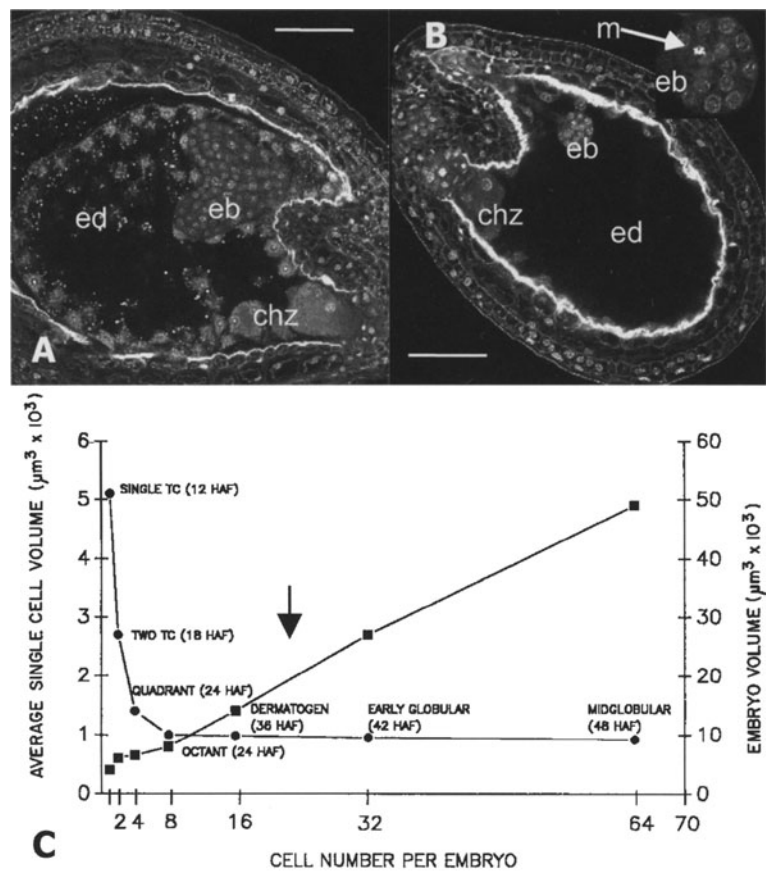
### 3.2

#### The *AtTOR* Gene

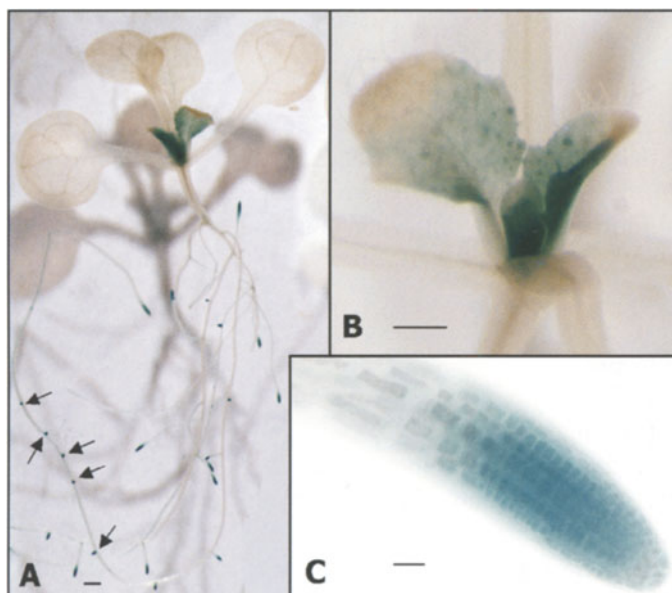
The *Arabidopsis* genome contains a single TOR gene (*AtTOR*) which encodes a protein displaying all the characteristics of TOR proteins including the highly conserved FRB, kinase and FATC domains, and the presence of HEAT repeats in its N-terminal two-thirds. HEAT repeats that are implicated in protein-protein interactions have also been found in the *Arabidopsis* microtubule-associated protein MOR1 (microtubule organization 1) (Whittington et al. 2001). The importance of those repeats was revealed by the finding that the two thermosensitive MOR1 mutants isolated from a screen for altered microtubule organization correspond to point mutations in a HEAT repeat.

The *AtTOR* activity is essential for plant growth, since *tor* null mutations lead to premature arrest of endosperm and embryo development (Fig. 2; Menand et al. 2002). This growth requirement for TOR is common in yeasts and animals. One of the TOR alleles is the result of an in-frame insertion of the GUS reporter gene, which thus allows a detailed and direct analysis of *AtTOR* expression in heterozygous plants (Menand et al. 2002). This reveals that the *AtTOR* gene is expressed in embryo, endosperm, primary meristems, and primordia (Fig. 3) but not in differentiated organs. This contrasts with the *mTOR* mRNA, which has been detected in all organs, except the heart, of 10-day-old mice embryos.

Several observations show that *AtTOR* is not needed for all types of cell division. First, the arrest of *tor-1* embryo development at the dermatogen stage correlates with a switch from divisions without cell growth to divisions coupled to cell growth (Mansfield and Briarty 1991; Fig. 2B, C). Indeed, during the early stages of *Arabidopsis* embryo development, there is little increase in total embryo volume, and the size of cells decreases during divisions (Fig. 2C). Second, genetic analysis show that divisions of the *tor* gametophyte can occur, leading to gamete formation (the alternation of generations where both haploid and diploid forms can divide is specific to plants). Third, the epidermal precursor cells of the stomates at the surface of the leaves divide without expressing TOR. All those divisions are the result of cleavages within a preexist-



**Fig. 2A-C** Phenotype of the *tor-1* mutant. Confocal section of a normal (A) and an aborted (B) seed from the same silique of a heterozygous *TOR/tor-1* mutant. A Zoom of the embryo is shown in the top right of B. *ed*, Endosperm; *eb*, embryo; *chz*, chalazal endosperm; *m*, metaphase. (From Menand et al. 2002, copyright 2002 National Academy of Science USA). C Change in cell (●) and embryo proper (■) volume during early embryogenesis 12–48 h after fertilization (HAF). The suspensor cells are not included in the data for embryo cell size or cell number. The arrow indicates the stage of arrest of TOR embryos development. TC, terminal cell. (From Mansfield and Briarty 1991, copyright 1991 National Research Council of Canada). Bar, 50  $\mu\text{m}$



**Fig. 3A–C** Expression pattern of *AtTOR*. Gus stainings were performed on the heterozygous TOR/TOR-1 mutant (10 days old) to localize the *AtTOR::GUS* fusion protein encoded by the *tor-1* allele. **A** Whole plant. *Arrows* show the emergence of secondary root meristems. **B** Zoom of leaves primordia. **C** Root meristem. *Bar*, 1 mm for **A** and **B**; 20  $\mu$ m for **C**

ing cell mass without net enlargement of the cytosol (Mansfield et al. 1991; Owen and Makaroff 1995; Zhao and Sack 1999).

Together these results suggest that *AtTOR* is not required for the process of plant cell expansion by vacuole enlargement and cell wall loosening, and that its activity is not essential to the mechanism of division itself. A simple hypothesis, in line with the knowledge of TOR functions in others organisms, is that *AtTOR* is required only for premitotic cytosolic growth.

### 3.3

#### Putative TOR Pathway-Related Genes in *Arabidopsis*

In addition to *AtTOR*, analysis of the *Arabidopsis* genome reveals the presence of other potential orthologs of the animal and yeast TOR pathway.

### 3.3.1

#### S6 Kinase

*Arabidopsis* possesses two orthologs of the p70 ribosomal protein S6 kinase. However, plants S6 kinases do not have the N-terminal domain of mammalian p70<sup>S6k</sup>, which is required for rapamycin sensitivity, and when expressed in mammalian cells, AtS6k2 is insensitive to rapamycin (Turck et al. 1998).

### 3.3.2

#### eIF4E-Binding Proteins

No homologs of the mammalian 4E-BPs are found in the *Arabidopsis* genome. However, the *Arabidopsis* lipoxygenase 2 (AtLOX2) has been reported to interact with eIF4E and eIF(iso)4E through a region similar to the conserved eIF4E-binding motif (Freire et al. 2000). Similarly, although no structural homologs of 4E-BPs exist in *S. cerevisiae*, two functional eIF4E-associated proteins (named p20 and Eap1p), containing the consensus eIF4E-binding domain and able to compete with eIF4G for binding to eIF4E, have been described (Cosentino et al. 2000). Implication of *EAP1* in the TOR pathway was demonstrated by the observation that its disruption confers partial resistance to rapamycin (Cosentino et al. 2000). Therefore, the absence of 4E-BPs homologs in *Arabidopsis* does not preclude a link between TOR and translation initiation. This link, and the implication of eIF4E-associated proteins, was conserved during evolution between yeast and mammals despite the recruitment of different proteins. Thus, its existence can thus be seriously hypothesized in plants.

### 3.3.3

#### TAP42 and Protein Phosphatases

In the budding yeast, and perhaps in mammals, TAP42 ( $\alpha 4$  in mammals) is an effector of the TOR pathway, which associates with the catalytic subunit of type 2A phosphatase (PP2A), leading to protein synthesis activation through an unknown mechanism. TAP46, the *Arabidopsis* homolog of TAP42, binds protein phosphatase 2A, and is thus a potential effector of *AtTOR* (Harris et al. 1999).

### 3.3.4

#### Cell-Cycle Genes: G1 Cyclins and p27<sup>Kip1</sup>

Several D-type cyclins exist in *Arabidopsis*, where they control the G<sub>1</sub>- to S-phase transition in response to cytokinin and sugar. Interestingly, the accumulation of cyclin D2 and D3 mRNAs, observed after addition of sucrose or cytokinins to sucrose-starved *Arabidopsis* cells, can be inhibited by protein phosphatase inhibitors (Riou-Khamlichi et al. 2000). This may suggest the involvement of *AtTOR*, since protein phosphatases are mediators of TOR downstream signalling pathway, in both mammals and budding yeast.

Another cell-cycle protein known to be downstream of mTOR in the signal transduction cascade is the CDK inhibitor p27<sup>Kip1</sup>. In *Arabidopsis*, homologs of p27<sup>Kip1</sup> form a family of seven genes. The overexpression of one member of this gene family leads to plants with fewer but larger cells compared to the wild type (De Veylder et al. 2001a).

### 3.3.5

#### Phosphatidylinositol-3 Kinases and PDK

Class I phosphatidylinositol-3 kinases (PI3-Ks) [the class of PI-3K able to generate PI(3, 4, 5)P<sub>3</sub>] have not yet been identified in *Arabidopsis*. However, a homolog of PDK1 (AtPDK1) has been identified from *Arabidopsis*, and was shown to contain a pleckstrin homology (PH) domain (PI(3, 4, 5)P<sub>3</sub> binding domains), to activate human PKB in vitro and to restore cell viability in *S. cerevisiae* mutants deleted of their PDK1 encoding genes (Deak et al. 1999). No Akt/PKB homologs are clearly identifiable in the *Arabidopsis* genome. Still, wortmannin, a PI3-K inhibitor in mammalian cells, inhibits the vesicular trafficking of proteins to the vacuole in tobacco cells but is not specific to PI3-K, since it also inhibits tobacco PI4-K (Matsuoka et al. 1995). Therefore, this drug may not be suitable for the study of a putative PI3-K pathway in plants.

### 3.3.6

#### Insulin-Like Signalling

Components of the insulin signalling pathway such as insulin-like receptors are not found in the *Arabidopsis* genome (The *Arabidopsis* Initiative 2000). An unidentified protein named ZmIGF (*Zea mays* insulin-like

growth factor) was purified by affinity chromatography with a bovine insulin antibody. This compound is able to accelerate germination and seedling growth. Moreover, treatment of maize hypocotyl by insulin or ZmIGF induces ribosomal S6 protein phosphorylation in a manner that is moderately affected by rapamycin (Garcia Flores et al. 2001). However, since TOR is the primary rapamycin target, an effect of rapamycin on maize TOR would have first to be demonstrated. Thus, the existence of an insulin-like signalling pathway in plants merits further investigation.

#### 4

### Conclusion and Perspective

The work of Menand et al. (2002) suggests that plants share with other eukaryotes an ancestral signalling pathway based on the TOR protein which supports the production of new cells in their meristematic proliferation zones. Postmeristematic growth, which accounts for most of the final plant size, is likely to be a different process, independent of AtTOR. Since multicellularity occurred independently in plants and animals (Baldauf et al. 2000), the nature of signals integrating the TOR pathway at the level of the whole organism is an interesting issue. Animal TOR proteins are involved in the sensing of amino acids and of the energetic status (ATP) of the cell, as well as in the relay of mitogen signals through the insulin receptor family. A role for TOR in relaying nutrient status to the plant meristematic zones can be hypothesized, either in the primary meristems of the shoot and of the root or in the environmentally dependent activation of root meristems by nutrients. Plant TOR may have been recruited during evolution by plant-specific signalling circuits involved in cell proliferation such as the ones triggered by phytohormones. The development of sophisticated genetic tools in model organisms of the plant kingdom, such as *Arabidopsis* or the moss *Physcomitrella patens*, will be essential for the comparative biology of the TOR pathway.

*Acknowledgements.* We wish to thank our colleagues in the Laboratoire du Métabolisme Carboné for fruitful discussions. B.M. is supported by an INRA-CEA doctoral fellowship.



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# TOR Action in Mammalian Cells and in *Caenorhabditis elegans*

X. Long<sup>1</sup> · F. Müller<sup>2</sup> · J. Avruch<sup>1</sup>

<sup>1</sup> Diabetes Research Laboratory, Departments of Molecular Biology,  
Land Medicine Massachusetts General Hospital and Department of Medicine,  
Harvard Medical School, 50 Blossom Street/Wellman 11, Boston, MA 02114, USA  
E-mail: [avruch@helix.mgh.harvard.edu](mailto:avruch@helix.mgh.harvard.edu)

<sup>2</sup> Institute of Zoology, University of Freiburg, Pérolles, 1700, Freiburg, Switzerland

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**Abstract** The p70 S6 kinase (p70 S6K) was the first signaling element in mammalian cells shown to be inhibited by rapamycin. The activity of the p70 S6K in mammalian cell is upregulated by extracellular amino acids (especially leucine) and by signals from receptor tyrosine kinases (RTKs), primarily through activation of the type 1A PI-3 kinase. The amino acid-/rapamycin-sensitive input and the PI-3 kinase input are co-dominant but largely independent, in that deletion of the amino-terminal and carboxy-terminal noncatalytic sequences flanking the p70 S6K catalytic domain renders the kinase insensitive to inhibition by both rapamycin and by withdrawal of amino acids, whereas this p70 S6K mutant remains responsive to activation by RTKs and to inhibition by wortmannin. At a molecular level, this dual control of p70 S6K activity is attributable to phosphorylation of the two p70 S6K sites: The Ptd Ins 3,4,5P<sub>3</sub>-dependent kinase1 (PDK1) phosphorylates p70 S6K at a Thr on

the activation loop, whereas mTOR phosphorylates a Thr located in a hydrophobic motif carboxyterminal to the catalytic domain. Together these two phosphorylations engender a strong, positively cooperative activation of p70 S6K, so that each is indispensable for physiologic regulation. Like RTKs, the p70 S6K appears early in metazoan evolution and comes to represent an important site at which the more ancient, nutrient-responsive TOR pathway converges with the RTK/PI-3 kinase pathway in the control of cell growth. Dual regulation of p70 S6K is seen in *Drosophila*; however, this convergence is not yet evident in *Caenorhabditis elegans*, wherein nutrient activation of the insulin receptor (InsR) pathway negatively regulates dauer development and longevity, whereas the TOR pathway regulates overall mRNA translation through effectors distinct from p70 S6K, as in yeast. The *C. elegans* TOR and InsR pathways show none of the cross- or convergent regulation seen in mammalian cells. The nature of the elements that couple nutrient sufficiency to TOR activity remain to be discovered, and the mechanisms by which RTKs influence TOR activity in mammalian cells require further study. One pathway for RTK control involves the tuberous sclerosis complex, which is absent in *C. elegans*, but of major importance in *Drosophila* and higher metazoans.

## 1

### TOR Action in Mammalian Cells

#### 1.1

##### Rapamycin-Sensitive and Insulin/Mitogen-Responsive Signaling Pathways Regulate the p70 S6 Kinase

Our interest in rapamycin developed in the course of an effort to understand the nature of the signal transduction pathways downstream of the insulin receptor (InsR) tyrosine kinase. In particular we were attempting to define the pathway by which insulin and other growth factors elicited a rapid increase in the serine phosphorylation of intracellular protein targets, specifically the ribosomal S6 polypeptide (Avruch et al. 1985; Nemenoff et al. 1986). We had recently purified (Price et al. 1989, 1990) and molecularly cloned (Banerjee et al. 1990; Grove et al. 1991) what appeared to be the major insulin/mitogen/stress-activated S6 kinase (S6K) from rat liver, an enzyme now known as p70 S6K alpha or S6K1. Parallel work by Maller, Erikson, and colleagues in the *Xenopus* oocyte had led

to the purification (Erikson and Maller 1985) and molecular cloning of several isoforms of the protein kinase Rsk (Jones et al. 1988; Alcorta et al. 1989) as the major S6K-activated protein during *Xenopus* oocyte maturation. In mammalian cells, both p70 S6K and Rsk are rapidly activated in response to mitogens, insulin, and active phorbol esters (Chen and Blenis 1990; Blenis and Erikson 1986), and both can phosphorylate S6 on 40 S ribosomal subunits in vitro to high stoichiometry (Wettenhall et al. 1992; Ferrari et al. 1991). Thus the identity of the kinase responsible for the insulin-induced phosphorylation of S6 in mammalian cells was unclear. Moreover, the nature of the signaling pathway(s) that connected the InsR to Rsk and the p70 S6K was also unknown. Sturgill, Maller, and colleagues had shown that Rsk could be directly phosphorylated and partially activated in vitro by an insulin-activated MAP kinase (probably corresponding to Erk2) (Sturgill et al. 1988). Although able to confirm this result, we were unable to show significant MAPK-catalyzed phosphorylation of p70 S6K (Price et al. 1990; Mukhopadhyay et al. 1992), raising the possibility that the signaling outflow from the InsR bifurcated upstream of the two candidate S6Ks. We became aware of rapamycin through Barbara Bierer; she and her colleagues had been characterizing the cellular physiology of the immunosuppressant agents rapamycin and FK506. These structurally related agents exhibited entirely distinct actions on T cells; FK506 (but not rapamycin) blocked a variety of responses initiated by activation of the T cell receptor (TCR), whereas rapamycin (but not FK506) strongly inhibited IL-2-induced proliferation (Bierer et al. 1990; Dumont et al. 1990). Nevertheless, the two agents bound to a common intracellular receptor, the peptidylprolylisomerase, FKBP12, and were mutually antagonistic (Dumont et al. 1990; Bierer et al. 1990). In collaboration with Bierer, we showed that rapamycin caused a profound inhibition in vivo of the basal and stimulated activity of p70 S6K, both endogenous and recombinant, and a concomitant inhibition of the 40S ribosomal S6 phosphorylation without any effect on the activity of Rsk (Price et al. 1992); similar results were reported by Blenis, Crabtree, and coworkers (Chung et al. 1992). These results identified p70 S6K as the physiological S6K in mammalian cells and indicated the existence of a signal transduction pathway, critical to proliferation of T cells (in response to IL-2) and H4 hepatoma cells (in response to insulin), that was completely independent of the pathway responsible for insulin activation of Rsk (subsequently shown to be the Ras/Raf/MAPK pathway; Avruch et al. 1994). Rapamycin inhibition of p70 S6K in vivo was

accompanied by the disappearance of the most slowly migrating p70 S6K polypeptide bands on SDS-PAGE, reflecting partial dephosphorylation. This finding, together with the observation that rapamycin, either alone or complexed with FKBP12, had no direct effect on p70 S6K activity in vitro, pointed to the likelihood that the target of rapamycin action was situated upstream of p70 S6K, and that rapamycin served to inhibit a p70 S6K-kinase, activate a p70 S6K-phosphatase, or both (Price et al. 1992).

The molecular identity of the “target of rapamycin” (TOR) was first established by a genetic approach in *S. cerevisiae*, by the cloning of a TOR2 mutant gene responsible for a dominant form of rapamycin resistance (Kunz et al. 1993; Cafferkey et al. 1993). The specific TOR2 mutation, Ser1972Arg, abrogates the binding of the rapamycin/FKBP12 complex to TOR, and thereby interdicts rapamycin sensitivity. The molecular structure of TOR established it as the founding member of the PI-kinase-related subfamily of kinases (PIKs). Within a year, several groups identified mammalian TORs (FRAP, RAFT, RAPT; Brown et al. 1994; Sabatini et al. 1994; Sabers et al. 1995; Chiu et al. 1994; Chen et al. 1994); moreover, a recombinant rapamycin-resistant mTOR mutant was shown to rescue coexpressed p70 S6K from inhibition by rapamycin, establishing mTOR/FRAP as the rapamycin-sensitive element upstream of p70 S6K (Brown et al. 1995; Hara et al. 1997).

## 1.2

### Relationship of the TOR Pathway to the Insulin/Mitogen Pathway in the Regulation of p70 S6K

Subsequent work in this and other laboratories established that the activation of p70 S6K by insulin and receptor tyrosine kinases (RTKs) required activation of the Type 1A PI-3 kinases (Chung et al. 1994), although perhaps not in *Drosophila* (Radimerski et al. 2002); thus p70 S6K is inhibited in vivo by low concentrations of PI-3 kinase (PI-3K) inhibitors (Cheatham et al. 1994), and activated by coexpression with constitutively active PI-3K variants (Weng et al. 1995b). Insulin activates p70 S6K by promoting a multisite Ser/Thr phosphorylation of the p70 S6K polypeptide. Thus, the insulin-stimulated, proline-directed multiple phosphorylation of an autoinhibitory *pseudosubstrate* (SKAIPS) domain in the p70 S6K $\alpha$  C-terminal flank does not itself cause activation (Banerjee et al. 1990; Price et al. 1991; Mukhopadhyay et al. 1992) but is neces-

sary to disengage the tail from the catalytic domain and enable access to the activating kinases (Weng et al. 1995a,b, 1998; Alessi et al. 1998). Activation of p70 S6K results from the simultaneous phosphorylation of a site on the "activation loop" in the catalytic domain, Thr252, and a second site situated in a hydrophobic motif located just carboxy-terminal to the catalytic domain, Thr412 (Pearson et al. 1995; Weng et al. 1998). Phosphorylation at either site alone engenders some activation, however the simultaneous phosphorylation at both sites exerts a strong, positively cooperative effect on p70 S6K activity, and is necessary for physiological activation in vivo. The inactivation of p70 S6K in vivo by rapamycin or wortmannin tracks very closely with the dephosphorylation of Thr412, whereas dephosphorylation of Thr252 in the activation loop decreases more slowly. Activation loop phosphorylation is catalyzed by PDK1 (Alessi et al. 1998), a kinase that binds avidly to the product of PI-3K, Ptd Ins 3,4,5P<sub>3</sub> (Alessi et al. 1997). Nevertheless, PDK1-catalyzed phosphorylation at p70 S6K Thr252 is unaffected by Ptd Ins 3,4,5P<sub>3</sub>, at least in vitro, but rather is strongly promoted by deletion of the p70 S6K C-terminal tail and by the phosphorylation (or substitution of Glu) at Thr412, which creates a high-affinity binding site for PDK1. The latter effect is very marked in vivo, inasmuch as mutation of Thr412 to Ala abolishes phosphorylation at Thr252. Reciprocally, mutation of Thr252 to Ala greatly decreases the phosphorylation at Thr412 during transient expression.

The identity of the kinase(s) responsible for phosphorylation at Thr412 in response to different stimuli in vivo remains uncertain. PDK1 itself can catalyze this phosphorylation in vitro, although only at 1%–2% of the rate of its phosphorylation at Thr252 (Balendran et al. 1999). Nevertheless, coexpression of p70 S6K with PDK1 results in substantial phosphorylation at Thr412, and ES cells bearing homozygous deletion of the PDK1 gene are unable to phosphorylate either Thr252 or Thr412 (Williams et al. 2000). Although this points to a role for PDK1 or a PDK1-activated kinase as the Thr412 kinase, the possibility remains that the absence of the strong positively cooperative effect of Thr252 phosphorylation on Thr412 phosphorylation accounts for the failure of Thr412 phosphorylation in PDK1 knockout ES cells. We purified a NIMA-like kinase, NEK6, from rat liver as the major p70 S6K Thr412 kinase; NEK6 can activate p70 in vitro and by coexpression, concomitant with Thr412 phosphorylation, in a manner synergistic with PDK1 (Belham et al. 2001). Nevertheless, NEK6 is not activated by insulin or inhib-



ited by either wortmannin or rapamycin; rather NEK6 is most active during mitosis, an interval when p70 S6K activity is at its nadir (Belham et al., in preparation). Finally, mTOR itself can directly phosphorylate in vitro p70 S6K, extracted in an inactive form from rapamycin-treated cells, at Thr412 and several sites in the carboxy-terminal tail, resulting in partial p70 reactivation (Burnett et al. 1998; Isotani et al. 1999); additional phosphorylation by PDK1 can then fully restore p70 S6K activity (Isotani et al. 1999).

This latter finding raises the possibility that mTOR acts as a direct p70 S6K in vivo, perhaps in response to activation of the InsR, and/or some nutrient/amino acid input. If TOR does act as a p70 S6K (Thr412) kinase, how does the TOR input relate to the PI-3K-directed pathway? Are these inputs disposed as sequential elements in a linear signaling pathway, or do they represent largely independent pathways that converge in the regulation of p70 S6K? A structure-function analysis of p70 S6K provided very strong support for the latter view (Weng et al. 1995a). p70 S6K contains a central catalytic domain surrounded by non-catalytic flanking regions. Deletion of both an acidic segment in the p70 S6K N-terminal flank (amino acids 29–46) and the entire C-terminal tail (i.e., including the SKAIPS domain) yields a p70 variant (p70 S6K $\Delta$ 2–46/ $\Delta$ CT104) whose low basal activity, like that of the parent wild-type p70 S6K, is activated by insulin and inhibited by PI-3K inhibitors. Nevertheless the activity of the p70 S6K $\Delta$ 2–46/ $\Delta$ CT104 is essentially completely resistant to rapamycin at concentrations that fully inhibit both the activity of wild-type p70 S6K in vivo and (in complex with FKPB12) the activity of the mTOR kinase in vitro. Moreover, although the maximal activity of the p70 S6K $\Delta$ 2–46/ $\Delta$ CT104 is considerably less than that of wild-type p70 S6K, the degree to which p70 S6K $\Delta$ 2–46/ $\Delta$ CT104 is activated by insulin and the magnitude of the insulin-stimulated phosphorylation at Thr412 in p70 S6K $\Delta$ 2–46/ $\Delta$ CT104 are completely unaffected by rapamycin. Thus, the insulin-activated Thr412 kinase is clearly rapamycin-insensitive, indicating that mTOR is not one of the elements necessary for insulin-stimulated phosphorylation and activation of p70 S6K $\Delta$ 2–46/ $\Delta$ CT104, and presumably wild-type p70 S6K (Weng et al. 1995a; Hara et al. 1998).

The most plausible explanation for the ability of rapamycin to promote the dephosphorylation of Thr412 and inactivation of p70 S6K is that active TOR somehow restrains a protein phosphatase that acts on p70 S6K (Fig. 1). In support of this view, Peterson et al. (1999) provided

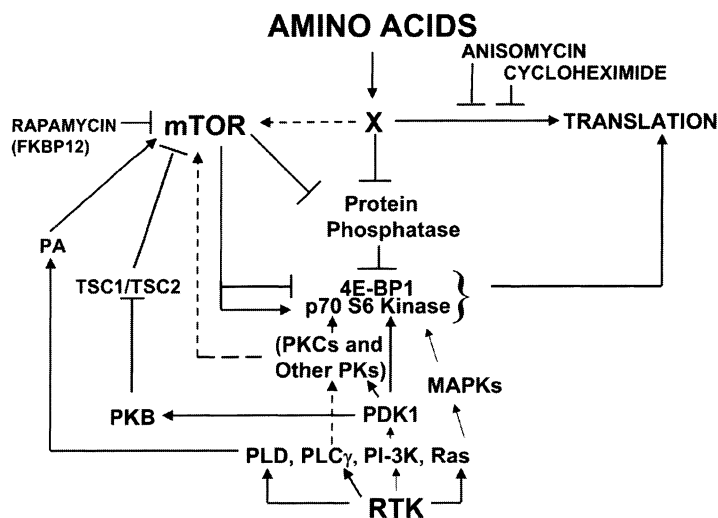


Fig. 1 Summary of mTOR signaling in mammalian cell culture

evidence that the PP2A catalytic subunit can associate with the wild type, but not the doubly deleted p70 S6K polypeptide. Specific information as to how mTOR regulates PP2A or other p70 S6K-phosphatases remains to be uncovered; a plausible hypothesis involves the participation of  $\alpha 4$  (Inui et al. 1998), a mammalian phosphatase regulatory protein that is homologous to TAP42, a rapamycin-sensitive yeast modulator of protein phosphatase activity (Di Como and Arndt 1996).

### 1.3

#### What Upstream Inputs Regulate TOR Activity?

The finding that mTOR, despite its ability to directly phosphorylate p70 S6K at Thr412 in vitro, is not required for the insulin activation of the p70 S6K $\Delta 2-46/\Delta$ CT104, raises several questions concerning the regulation of mTOR kinase activity: Is this activity constitutive or regulated, and if regulated, what are the major regulators? Several studies demonstrate that mTOR kinase activity toward 4E-BP measured in vitro can be stably activated by treatment of cells with insulin, or more robustly, with the neurotrophins CNTF (Yokogami et al. 2000) or BDNF (Takei et al. 2001). In *S. cerevisiae*, a unicellular organism that lacks RTKs, TOR is a

crucial determinant of cell proliferation; addition of rapamycin or inactivation of both TOR genes results in cell cycle arrest in early G<sub>1</sub> with a phenotype that strongly resembles that elicited in response to nutrient deprivation (G<sub>0</sub>) (Barbet et al. 1996). Importantly, overall mRNA translation is reduced by 90%, although some mRNAs, e.g., those encoding heat-shock proteins or ubiquitin, continue to be translated. In parallel to its regulation of mRNA translation, TOR also regulates transcription, autophagy, and the actin cytoskeleton in *S. cerevisiae* (Schmelzle and Hall 2000). The similarity of TOR-deficiency phenotype to that elicited by nutrient deprivation suggested that TOR participates in a nutrient-sensing pathway in yeast. This paradigm, together with the results of Blommaert et al. (1995) and the apparent independence of the mTOR input into p70 S6K from that provided by the insulin signal transduction pathway, led us to consider whether TOR retained a role in the nutrient regulation of mRNA translation in metazoans. We therefore examined, in a variety of cultured mammalian cells, whether the phosphorylation of p70 S6K and 4E-BPs, two TOR targets not found in *S. cerevisiae*, is regulated by the availability of amino acids (Hara et al. 1998). Withdrawal of ambient amino acids results in a rapid dephosphorylation of p70 S6K and 4E-BP, rendering them entirely unable to undergo phosphorylation/activation in response to insulin. Nevertheless, amino acid withdrawal has no major effect on the insulin activation of the core elements of the insulin signaling pathway, i.e., IRS tyrosine phosphorylation, PI-3K activation, PKB/Akt, and MAPK. The pattern of responses to amino-acid withdrawal is essentially identical to that induced by rapamycin. Most importantly, the insulin-responsive, wortmannin-sensitive, but rapamycin-resistant p70 S6K $\Delta$ 2-46/ $\Delta$ CT104 variant also proved to be resistant to dephosphorylation/deactivation in response to amino-acid withdrawal. This result indicated that both mTOR and amino-acid sufficiency regulate p70 S6K phosphorylation/activation through the same regulatory element, presumably the TOR-inhibitable protein phosphatase. Evidence is lacking however as to whether amino-acid regulation of this putative p70 S6K-phosphatase occurs through the intermediation of TOR. We were unable to rescue wild-type p70 S6K from amino-acid withdrawal by coexpression with a rapamycin-insensitive mTOR (Ser2035Thr) mutant, although positive results in such an experiment have been reported by others (Iiboshi et al. 1999).

Further evidence of the similarity of TOR deficiency to nutrient deprivation comes from the phenotype of TOR deficiency in *Drosophila*

(Zhang et al. 2000; Oldham et al. 2000), which induces a larval arrest which resembles that resulting from amino-acid deprivation (Britton and Edgar 1998) in several respects, namely reduced nucleolar size, lipid vesicle aggregation in the larval fat body, and a selective arrest of growth in the endoreplicating tissues that can be bypassed by overexpression of cyclin E. Notably, the developmental arrest of DmTOR deficiency can be partially overcome by overexpression of a mutant, partially active version of p70 S6K (wherein four phosphorylation sites in the SKAIPS domain are changed to Asp), enabling TOR-deficient larvae to progress (somewhat) to pupae (Zhang et al. 2000). The deficiency of DmTOR is epistatic to deficiency of PTEN in the regulation of cell size (Oldham et al. 2000), indicating that, as in cultured mammalian cells, the TOR signal converges with that of PI-3K, and is necessary for PI-3K input to function as a positive regulator of cell growth. Thus whereas TOR exerts primary hegemony over mRNA translation in the unicellular organism *S. cerevisiae*, this function in *Drosophila* and higher metazoans is shared with PI-3K, the primary transducer of insulin signals. Nevertheless, TOR deficiency/rapamycin is dominant over the PI-3K input, at least in regard to cell growth. There is likely to be extensive intracellular cross-regulation between the nutrient-driven TOR pathway and RTKs, especially those in the InsR family, as the latter are the primary determinants of cell size (and number) during embryogenesis (independent of patterning) and of growth and the interorgan partitioning of nutrients after birth.

## 2

### TOR Action in *C. elegans*

#### 2.1

##### TOR Function in *C. elegans*

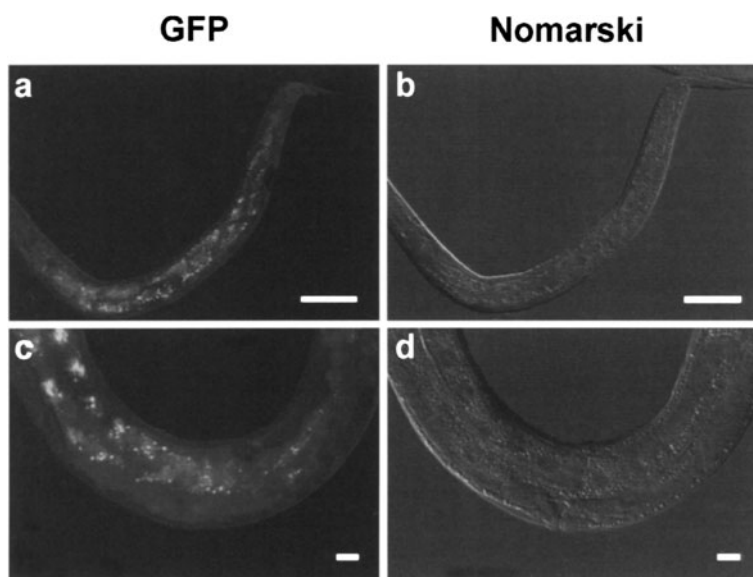
To gain further insight in a metazoan into the relationship between the InsR and TOR pathways, as well as the identity of additional components of the TOR pathway, we recently undertook an examination of TOR function in the nematode *Caenorhabditis elegans*, taking advantage of its short generation time and the facile genetic and molecular genetic tools developed for this metazoan organism (Long et al. 2002). Our initial analysis indicates that *C. elegans* TOR is necessary for larval development, through its role as a major upstream regulator of overall mRNA

translation. A deficiency of CeTOR, however, does not promote dauer development, and thus CeTOR is not a component of the pathway by which the *C. elegans* InsR/PI3K/PDK1 pathway controls development.

A *C. elegans* TOR homolog (CeTOR) was identified by searching the worm genomic database for homologs of phosphatidylinositol kinase (PIK)-related proteins. CeTOR protein contains 2,697 amino acids and has significant similarity to the yeast TORs (33% identity) and the mammalian TOR (39% identity). Functional domains and residues critically involved in TOR's FKBP12-rapamycin binding (Chen et al. 1995; Chiu et al. 1994; Cafferkey et al. 1993; Helliwell et al. 1994; Stan et al. 1994; Zheng et al. 1995), kinase domain activation (Vilella-Bach et al. 1999), and phosphotransferase reaction (Hanks et al. 1988), etc. are all conserved in CeTOR (Long et al. 2002). Transgenic expression of CeTOR::GFP fusion protein indicates that zygotic TOR expression starts in the comma-stage embryos, and TOR is expressed in all major tissues and organs in all subsequent developmental stages (Fig. 2). Whole-mount embryo in situ hybridization experiments show that CeTOR RNA is present in the cytoplasm of all cells starting with the one-cell-stage embryos, indicating that CeTOR mRNA is also maternally provided (data not shown).

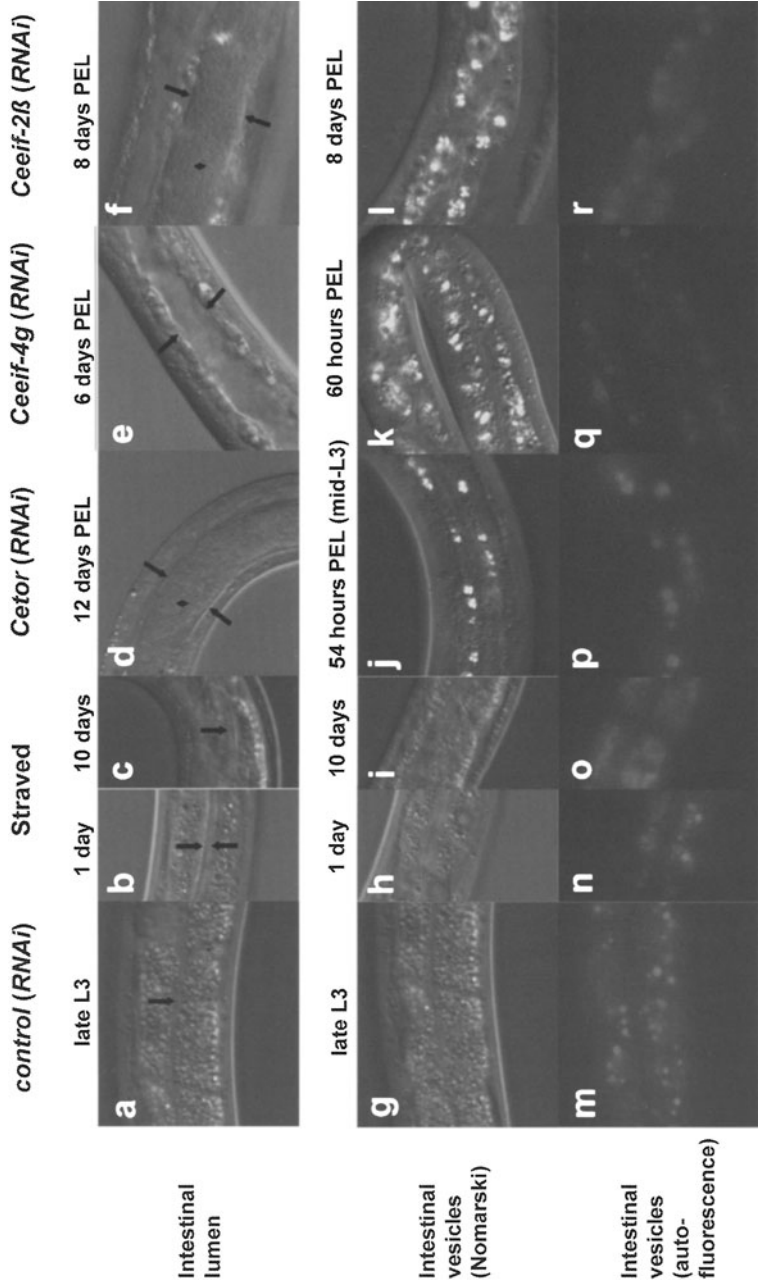
Rescue experiments with a cosmid containing the entire CeTOR coding sequence followed by DNA sequence analysis identified alleles of *let-363* complementation group as harboring mutations in the *CeTOR* gene. Alleles *h111*, *h131*, and *h114* contain point mutations leading to stop codons after amino acids 104, 906, and 2398, respectively (Long et al. 2002). Genetic evidence suggests that these three alleles are null alleles.

Phenotypes associated with CeTOR deficiency were characterized in both CeTOR mutant (allele *h111*) animals and CeTOR RNA interference (RNAi)-treated animals. RNAi was introduced by feeding worms bacteria expressing double-stranded RNA (dsRNA) corresponding to the gene-of-interest (Fraser et al. 2000). RNAi through feeding was initiated in L3- to L4-stage larvae; progeny (F1) from RNAi-raised animals were fed the dsRNA-producing bacteria as the sole food source from the time of hatching, allowing continued strong RNAi effect in the F1 generation. Introduction of RNAi through feeding has proven to be as effective as the conventional method of injecting synthesized dsRNA into the developing worm gonad (e.g., Fraser et al. 2000) but much less laborious, as it obviates the need to synthesize dsRNA. Identical phenotypes were observed for the CeTOR mutant animals and RNAi-treated animals.



**Fig. 2a–d** CeTOR expression patterns during development and in adult. **a, c** CeTOR::GFP expression patterns. Note that the *let-363/tor::gfp* translational fusion construct harbors a nuclear localization signal in the vector. **b, d** Animals in (a) and (c) under Nomarski optics. **a, b** CeTOR::GFP expression in a wild-type late L3 stage animal. CeTOR::GFP is expressed in many cells and tissues that are affected by the *let-363* mutation. **c, d** CeTOR::GFP expression in a young adult. Shown is the middle part of the body. Anterior is to the left. Scale bar, 10  $\mu$ m

CeTOR deficiency, whether by mutation or RNAi, results in several characteristic abnormalities. (1) CeTOR-deficient animals have significantly delayed somatic and gonadal development and eventually arrest at late L3 gonadal stage (data not shown; Long et al. 2002). (2) CeTOR-deficient larvae exhibit an increase in the size and number of lipid-laden hypodermal vesicles (data not shown; Long et al. 2002). (3) The most striking feature is a very characteristic intestinal phenotype. Beginning early in the L3 stage, the intestinal cells of CeTOR-deficient animals exhibit large, highly refractile, and autofluorescent “lipofuscin granules” (Fig. 3j, p) that have previously been identified as intestinal lysosomes (Clokey and Jacobson 1986; White 1988; Kostich et al. 2000; Matyash et al. 2001). These vesicles become labeled with Neutral Red, a lysosomal marker, when the dye is introduced by feeding (data not shown; Long et al. 2002). In contrast to the increased size of “lipofuscin granules”, the



usual nonrefractile, nonautofluorescent intestinal vesicles (lipid droplets and protein/carbohydrate storage vesicles; White 1988; Kostich et al. 2000) decrease dramatically in number (Fig. 3j), accompanied by a progressive decrease of intestinal cytoplasmic volume. The decrease in both the nonrefractile intestinal vesicles and in the nonparticulate intestinal cytosol reduces the intestinal volume to a thin layer in which only the large autofluorescent vesicles and nuclei are visible. This is paralleled by a progressive enlargement of intestinal lumen; by 6–8 days post-hatching, the lumen is grossly dilated and filled with undigested or half-digested bacterial clumps, presumably a consequence of the intracellular atrophy and resultant loss of digestive and or secretory function of the intestine (Fig. 3d). It is likely that the slowing and ultimate arrest of de-



**Fig. 3a–r** CeTOR-deficient animals demonstrate severe intestinal atrophy, which can be phenocopied by deficiency in general translation initiation factors. **a–f** Intestinal lumen morphology. **a** Intestinal lumen (*arrow*) in a late L3 N2 raised on control RNAi. **b** Intestinal lumen (*arrows*) in an N2 arrested at late L3 stage, 1 day after starvation initiation. **c** Intestinal lumen (*arrow*) in an N2 arrested at late L3 stage, 10 days after starvation initiation. **d** The dramatically enlarged intestinal lumen (*arrows*) of a 12-day post-egg-lay (PEL) N2 F1 arrested on CeTOR RNAi. A diamond indicates undigested or half-digested bacteria filling the intestinal lumen. **e** A 6-day PEL N2 F1 raised on CeeIF-4G RNAi has dramatically decreased intestinal cytoplasmic volume and enlarged lumen (*arrows*). **f** An 8-day PEL N2 F1 raised on CeeIF-2 $\beta$  RNAi has dramatically decreased intestinal cytoplasmic volume and enlarged lumen (*arrows*). A diamond indicates the undigested and half-digested bacterial clumps filling the lumen. **g–l** Intestinal vesicles. **m–r** DAPI channel autofluorescence images corresponding to **g–l**. **g, m** Intestinal vesicles in a late L3 N2 raised on control RNAi. **h, n** Intestinal vesicles in an N2 arrested at late L3 stage, 1 day after starvation initiation. Note the dramatic decrease in total intestinal vesicle number and virtually no increase in the autofluorescent vesicle size (**n**). **i, o** Intestinal vesicles in an N2 arrested at late L3 stage, 10 days after starvation initiation. In **i**, note the dramatic decrease in total nonrefractile, nonautofluorescent intestinal vesicle number compared to **h**. The autofluorescence signals in **o** appear blurry, mainly due to increased hypodermal granule density at these times (data not shown; Long et al. 2002). **j, p** The middle section of a mid-L3 stage N2 raised on CeTOR RNAi. Anterior is to the left. In **j**, note the large refractile intestinal vesicles and the decreased number and size of nonrefractile intestinal vesicles (compared to **g**). **k, q** A 60-h PEL N2 F1 raised on CeeIF-4G RNAi has large, refractile, and autofluorescent intestinal lysosomes and decreased number of nonrefractile intestinal vesicles. **l, r** An 8-day PEL N2 F1 raised on CeeIF-2 $\beta$  RNAi has large, refractile, and autofluorescent intestinal lysosomes and decreased number of nonrefractile intestinal vesicles



velopment results at least in part from defective nutrient absorption, inasmuch as starvation per se results in a rapid developmental arrest. The phenotype of CeTOR-deficient animals nevertheless differs substantially from that of larvae arrested at L3 by starvation during L2 or early L3; starved L3 animals exhibit neither large, highly refractile and autofluorescent intestinal lysosomes nor progressive loss of intestinal cytoplasm (and the accompanying luminal enlargement) (Fig. 3h, i, n, o). It appears that CeTOR deficiency leads to the marked upregulation of a process that “consumes” intestinal cytoplasm (probably lysosome-mediated degradation) beyond that triggered by starvation alone; alternatively this process may be actively suppressed in starved animals. In addition, whereas L3 animals starved for as long as 12 days can resume development and produce progeny when provided with food, CeTOR-deficient animals arrested for 7–8 days generally remain arrested or die even after being transferred to food (Long et al. 2002).

Inactivation of the *C. elegans* insulin receptor homolog DAF-2 or its downstream effectors (i.e., PI3K/PDK1/PKB) causes dauer-stage arrest (Morris et al. 1996; Kimura et al. 1997; Paradis and Ruvkun 1998; Paradis et al. 1999). Although TOR has previously been implicated in the insulin/IGF-1 network in studies performed in mammalian cells and *Drosophila* (Raught et al. 2000; Scott et al. 1998; Zhang et al. 2000), deficiency of TOR in *C. elegans* does not promote dauer development, inasmuch as the phenotypes of CeTOR-deficient animals are distinct from dauers in all major characteristics. Thus, CeTOR does not participate in the pathway by which the *C. elegans* InsR pathway inhibits dauer development; whether the *C. elegans* InsR regulates CeTOR output, however, cannot be determined from data currently available.

## 2.2

### Studies of Candidate CeTOR Downstream Effectors Reveal Translational Control as the Major Physiological and Developmental Target of CeTOR

In an effort to determine whether any of the TOR effectors identified in other organisms could account for some or all of the phenotypes of TOR deficiency in *C. elegans*, we attempted to identify the *C. elegans* homologs of these elements and examine the effects of inhibiting their expression through RNAi. We first inquired as to whether a deficiency in the activity of the p70 S6K accounts for any portion of the major phenotypes of CeTOR deficiency (as is reported for *Drosophila*). A *C. elegans*

p70 S6K homolog (Cep70) was identified through database searches (Long et al. 2002). F1s of wild-type animals raised on Cep70 RNAi did not however exhibit any of the striking phenotypes caused by CeTOR deficiency, but rather a distinct set of phenotypes, including a slight developmental delay, a slightly reduced final body size, and larger nonrefractile intestinal vesicles in adults (data not shown). Thus, the major phenotypes of CeTOR deficiency are not due to a deficiency of Cep70 S6K function.

The *S. cerevisiae* TOR proteins have been suggested to signal in part through the TAP42 polypeptide, which regulates the SIT4 and PP2A phosphatase activities (DiComo and Arndt 1996; Beck and Hall 1999; Jiang et al. 1999; Cutler et al. 2001). TIP41 negatively regulates the TOR pathway by binding and inhibiting TAP42 (Jacinto et al. 2001). We have identified the homologs for TAP42, SIT4, and TIP41 in the *C. elegans* genome (Long et al. 2002). F1 adults raised on CeTAP42 RNAi, CeTIP41 RNAi, and a mix of CeSIT4.1 and CeSIT4.2 RNAs have fertility defects, as evident from decreased total egg-lay number (CeTAP42 RNAi, 90%–95% decrease; CeTIP41 RNAi, 80%–90% decrease; CeSIT4.1, 70%–80% decrease; a mix of CeSIT4.1 and CeSIT4.2 RNAs, ~ 90% decrease) and/or high F2 embryonic lethality (CeTIP41 RNAi, ~80% lethal; the mix of CeSIT4.1 and CeSIT4.2 RNAs, 80%–90% lethal), but no other visible abnormality. Moreover, F1s of CeTOR mutant animals raised on any of these RNAs appeared identical in every aspect to those of CeTOR mutant animals raised on control RNAi. We conclude that the major somatic phenotypes of CeTOR deficiency do not result from a disruption of the TAP42-SIT4-TIP41 signaling branch downstream of TOR; in *C. elegans*, this branch appears to be involved mainly in gonad development and function.

We sought to determine whether interference with the components of the eIF-4F complex underlies the CeTOR-deficiency phenotypes. Search of the worm genome failed to identify a structural homolog of 4E-BP1. Conversely, five *C. elegans* proteins with significant homology to the human eIF-4E have been identified (Jankowska-Anyszka et al. 1998; Keiper et al. 2000; Amiri et al. 2001), making eIF-4E a poor candidate for analysis by RNAi. Disrupting the worm eIF-4A homolog through RNAi feeding caused no phenotypes in P0 animals and resulted in only reduced fertility in F1 adults (brood size is ~30% of that of animals raised on control RNAi).

The *C. elegans* genome contains a single homolog of the scaffold protein, eIF-4G (CeeIF-4G), which functions to assemble the 43S ribosomal preinitiation complex together with 5' MeGTP-capped mRNAs, through its ability to bind eIF-3 and eIF-4E, respectively. Disrupting CeeIF-4G through RNAi feeding caused no morphological phenotypes in P0 animals. However, the F1 hatchlings were severely delayed in gonadal and somatic development and arrested at L3 5–6 days post-egglay (PEL) (data not shown; Long et al. 2002). F1s raised on CeeIF-4G RNAi also contain large, refractile, and autofluorescent intestinal lysosomes starting at L2 (Fig. 3k, q), and exhibit a progressive decrease in the number of nonrefractile intestinal vesicles as well as in overall intestinal cytoplasmic volume (Fig. 3q, e), accompanied by an enlargement of the intestinal lumen. By 6 days PEL, intestinal cells are reduced to a thin layer outlining the lumen (Fig. 3e), an appearance closely resembling the severe intestinal atrophy in CeTOR-deficient animals. Furthermore, hypodermal granule size is progressively increased in F1s raised on CeeIF-4G RNAi, compared to equivalent stage F1s raised on control RNAi (data not shown; Long et al. 2002).

In view of the modest phenotype induced by deficiency of eIF-4A, we inferred that the major changes resulting from eIF-4G deficiency reflected a defect in overall mRNA translation rather than a selective deficiency in the expression of mRNAs with complex structures in their 5' untranslated segment. This conclusion was supported by the finding that disruption (through RNAi feeding) of the *C. elegans* homologs of the general translational initiation factors eIF-2 $\alpha$  (CeeIF-2 $\alpha$ ) and eIF-2 $\beta$  (CeeIF-2 $\beta$ ) produced phenotypes identical to those produced by CeeIF-4G deficiency (Fig. 3f, l, r; data not shown). The close similarity between the phenotypes caused by interference with the expression of CeTOR and the general translational initiation factors is unlikely to be fortuitous; although larval stage arrest/lethality can be caused by a disruption in hundreds of genes (Fraser et al. 2000), the appearance of intestinal atrophy and large, refractile autofluorescent intestinal lysosomes is a very characteristic and specific phenotype. Thus, as previously observed in *S. cerevisiae*, CeTOR is an upstream regulator of global mRNA translation, in contrast to its more limited role in mammalian systems.

Inasmuch as TOR negatively regulates autophagy (Noda and Ohsumi 1998; Blommaert et al. 1995), the appearance of large, refractile, and autofluorescent intestinal lysosomes in CeTOR-deficient worms led us to suspect that the intestinal atrophy might reflect increased autophagic ac-

tivity. However, feeding RNAi directed at the *C. elegans* homologs for APG12 and APG7, which are both essential for autophagy in yeast (Mizushima et al. 1998; Kim et al. 1999), caused no detectable phenotypes in wild-type background worms and did not affect any aspect of the CeTOR-deficient mutant animals. Specifically, the appearance of the large and refractile intestinal lysosomes in CeTOR-deficient animals is not affected to any extent by APG12 and APG7 disruption. Therefore, the role of autophagy in the major intestinal phenotypes seen with CeTOR inactivation is as yet uncertain.

### 3

#### Perspective

In *Drosophila*, disruption of InsR and its downstream molecules (*chico*, Akt/PKB, etc.) leads to fewer and smaller cells, whereas deficiency of p70 S6K results in smaller cells with no change in cell number. Moreover, the IGF/IGF-1R system is a major determinant of embryonic growth in mammals (independent of patterning). These findings establish a major role of IGF/insulin signaling in the determination of cell growth during development (Chen et al. 1996; Montagne et al. 1999; Huang et al. 1999; Verdu et al. 1999; Bohni et al. 1999; Goberdhan et al. 1999; Stocker and Hafen 2000; Brogiolo et al. 2001; Radimerski et al. 2002; Shioi et al. 2002). *Drosophila* TOR mutants are also deficient in growth and proliferation during larval development (Zhang et al. 2000; Oldham et al. 2000); DmTOR is necessary for the increase in cellular growth caused by activation of the PI-3K signaling pathway, at least in part by its ability to control the activity of DmS6K. In other words, DmTOR is necessary during fly development to enable the stimulation of cellular and organismal growth in response to the InsR signaling output. Recent work indicates that *Drosophila* p70 S6K activity, while dependent on PDK1, is independent of PI-3K (Radimerski et al. 2002). We speculate that DmTOR activity is responsive primarily to signals reflecting nutrient sufficiency; when these are insufficient, the fall in TOR activity overrides the InsR stimulation of "growth", in part by deactivation of p70 S6K. Unfortunately, however, the biochemical functions of the p70 S6K relevant to "growth" remain elusive.

In *C. elegans*, nutrient insufficiency during early larval development (L1) acts via a neuronally generated signal (an inhibitory insulin-like ligand; Pierce et al. 2001) to downregulate the InsR pathway, resulting in

the disinhibition of an alternative developmental program leading to a diapause state known as the dauer larvae (Morris et al. 1996; Kimura et al. 1997; Paradis and Ruvkun 1998; Paradis et al. 1999). Partial loss-of-function mutants of *C. elegans* InsR, PI3K, PKB, etc. do not show defects in organismal or cellular growth (G. Ruvkun, personal communication). Moreover, the negative impact of p70 S6K deficiency on the growth of *C. elegans*, although detectable, is far less marked than in *Drosophila* (Long et al 2002). Our analysis thus far indicates that *C. elegans* TOR resides in a pathway completely separate from the "canonical" insulin pathway; CeTOR does not participate in regulation of diapause, but rather controls global mRNA translation, a situation similar to that prevailing in the single-cell eukaryote, *S. cerevisiae*. It will be important to determine whether the *C. elegans* InsR (Daf2) regulates CeTOR function, as is likely in mammalian cells and perhaps in flies, as well as to uncover the elements (if any) that regulate CeTOR activity as well as those which mediate TOR signaling to translation in *C. elegans*.

**Acknowledgements.** We thank Dr. Gary Ruvkun for his expert advice and for generously allowing us to use his laboratory equipment, and members of the Ruvkun lab for technical instruction and worm morphology identification. Ann M. Rose, Carmen Spycher, and Z. Stanley Han's initial contribution to the *C. elegans* part of the project is sincerely acknowledged. We also thank Yenshou Lin for help with figure preparations, Jeanette Prendable for help with manuscript preparation, and other members of the Avruch lab for advice on molecular techniques. Some of the strains used in this study were provided by the Caenorhabditis Genetics Center (CGC), which is funded by the NIH National Center for Research Resources (NCRR). This work was supported by Swiss National Science Fund 31-40776.94 and 31-56953.99 (to F.M.) and NIH grants DK17776 and CA73818 (to J.A.).

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# Genetic Analysis of TOR Signaling in *Drosophila*

T. P. Neufeld

Department of Genetics, Cell Biology, and Development, University of Minnesota,  
6–160 Jackson Hall, 321 Church Street S.E., Minneapolis, MN 55455, USA  
E-mail: [neufeld@med.umn.edu](mailto:neufeld@med.umn.edu)

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**Abstract** Over a 4-day period of development, *Drosophila* larvae undergo a roughly 1,000-fold increase in mass. This impressive growth requires a continuous source of dietary protein; in the absence of amino acids, growth is arrested and various larval tissues display characteristic cell-cycle, metabolic, and structural changes. Mutations in the *Drosophila* target of rapamycin (*dTOR*) gene result in strikingly similar phenotypes, suggesting that dTOR acts in a signaling pathway responsive to nutrient availability. Genetic epistasis experiments indicate that dTOR is also required for cell growth in response to insulin and PI3K signaling, and that S6K activation can partially rescue dTOR loss of function. Thus dTOR has roles in both nutrient- and growth factor-mediated signaling, and may act to coordinate the activities of these pathways during development. Here we describe the use of mutations in *dTOR* to dissect its

role in various signaling events, to gain insight into TOR protein structure, and to identify novel factors involved in TOR signaling.

## 1

### Introduction

During animal development, precise regulation of cell growth and proliferation is critical to attain normal body size and shape (for review, see Conlon and Raff 1999). Classical embryological studies have focused on two distinct mechanisms of such regulation (reviewed in Bryant and Simpson 1984). First, information intrinsic to individual organs or appendages is required for autonomous growth control. For example, imaginal discs (the primordia of most adult structures in *Drosophila*) will grow to their normal mature size when transplanted into an adult host. This type of regulation appears closely tied to the morphogenic patterning systems which give shape and pattern to particular organs. Second, systemic regulators such as growth factors and hormones can govern growth of all tissues simultaneously throughout an organism, and thus play an important role in maintaining normal body proportions.

In addition to these genetically controlled mechanisms, growth in most organisms is also highly sensitive to environmental conditions. Factors such as nutrition and temperature can substantially alter both the rate and final extent of growth. Environmental and genetic factors can interact in complex ways, and therefore cellular components that function to integrate environmental cues with genetic signaling pathways are likely to be especially critical for proper control of growth during development.

The TOR family of proteins is unique in having the potential to act at this nexus between environmentally and genetically controlled signaling pathways. TOR proteins were originally identified in yeast, where they act to regulate a number of cellular processes in response to the quality of nitrogen and carbon sources (Heitman et al. 1991; Beck and Hall 1999; Kuruvilla et al. 2001). In multicellular organisms, TOR homologs have apparently retained their function as cell-autonomous nutrient sensors, but have also gained the capacity to control intercellular signaling through effects on the insulin/phosphoinositide kinase (PI3K) pathway (reviewed in Schmelzle and Hall 2000). TOR proteins may thus act in higher eukaryotes as part of a checkpoint mechanism that ensures that

intercellular growth signals are appropriate for a given nutritional status.

This review will discuss the role of TOR signaling in *Drosophila*, with an emphasis on recent findings from genetic studies. Disruption of TOR in developing flies results in phenotypes similar to those caused by nutrient deprivation or by perturbations of insulin signaling. Studies of *dTOR* mutants are revealing new insights into the role of TOR in developmental and adult processes, and are shedding light on structural aspects of the TOR protein. Furthermore, mutations in TOR are being exploited to identify genetically interacting components of the TOR signaling pathway.

## 2

### Identification of Mutations in *dTOR*

The *Drosophila* genome encodes a single TOR-related protein, named dTOR, which displays extensive sequence similarity to both yeast and mammalian TOR proteins (Oldham et al. 2000; Zhang et al. 2000). Mutations in *dTOR* were generated by a reverse genetic approach from an existing collection of P-element insertions (Zhang et al. 2000). Imprecise mobilization of an element from the 5' UTR of *dTOR* gave rise to deletions which eliminate the translation start site and much of the *dTOR* coding sequence, resulting in null *dTOR* alleles. Concurrently, Oldham et al. (2000) identified alleles of *dTOR* in an elegant screen for growth mutants targeted to the developing eye. In addition, a series of *dTOR* point mutants have recently been identified in screens for EMS-induced mutations that fail to complement a null *dTOR* allele (see below). Together, strains of flies carrying these mutations constitute powerful tools to address the normal role of dTOR in development and physiology.

Mutations that completely abolish dTOR expression or function result in an arrest during early larval development, just prior to the stage of exponential growth (Oldham et al. 2000; Zhang et al. 2000). A similar growth-arrest phenotype has been observed in a number of insulin/PI3K signaling mutants such as Dp110 and Dp60 (Weinkove et al. 1999), as well as in wild-type larvae grown in the absence of amino acids (Britton and Edgar 1998). These phenotypic similarities have prompted detailed comparisons of the cellular effects of *dTOR* mutants with insulin pathway mutants and with the effects of starvation.

## 3

**Phenotypic Comparisons with Insulin/PI3K Pathway Mutants**

Disruption of the insulin/PI3K signaling pathway in *Drosophila* has been shown to cause a reduction in cell and organ growth (Chen et al. 1996; Leever et al. 1996; Bohni et al. 1999; Goberdhan et al. 1999; Huang et al. 1999; Verdu et al. 1999; Gao et al. 2000; Oldham et al. 2000; Zhang et al. 2000; Gao and Pan 2001; Potter et al. 2001; Scanga et al. 2001; Radimerski et al. 2002; Rintelen et al. 2002). Generally, this reduced growth is accompanied by a decrease in cell size, and in some cases by an extended G<sub>1</sub> phase of the cell cycle and a reduced rate of cell proliferation. As described below, mutations in *dTOR* cause similar phenotypes and display genetic interactions with insulin/PI3K signaling mutants.

Analyses of multiple cell types in the fly have shown that dTOR is required to achieve normal cell size. For example, adult bristle sheath cells are up to 50% smaller in the absence of dTOR. Consistent with a proposed role for TOR in ribosome biogenesis, reduced bristle size is a hallmark of a class of mutants known as Minutes, which result from mutations in ribosomal proteins (Lambertsson 1998). In addition, cells actively undergoing mitotic proliferation are also decreased in size in *dTOR* mutants, as shown by FACS analysis of imaginal discs containing mosaic clones of cells lacking dTOR (Zhang et al. 2000). Thus, the observed decrease in adult cell size appears to stem from both a reduction in postmitotic growth during metamorphosis and a smaller size of mitotically proliferating cells.

FACS analysis has also been used to demonstrate that dTOR is required for normal cell-cycle progression in mitotically active tissues during larval development. Loss of dTOR results in an increased proportion of imaginal disc cells in the G<sub>1</sub> phase of the cell cycle, indicating that G<sub>1</sub> is extended in *dTOR* mutants (Zhang et al. 2000). This delay in G<sub>1</sub> likely results from a decreased rate of accumulation of factors required for G<sub>1</sub>/S progression. Indeed, the levels of one such factor, cyclin E, were found to be significantly reduced in *dTOR* mutant larvae (Zhang et al. 2000). Moreover, overexpression of cyclin E can drive arrested *dTOR* mutant cells into S phase (see below).

Thus, effects of *dTOR* mutants on cell size, cell-cycle phasing, and cell proliferation are similar to the effects of mutations that inactivate or reduce insulin/PI3K signaling. Note, however, that such mutations may have either more severe (as in the case of *dAkt* or *Inr*), or less severe

(*dS6K*) developmental consequences than those of *dTOR* mutants, likely reflecting the complex interactions and multiple inputs, outputs, and branchpoints of this signaling system.

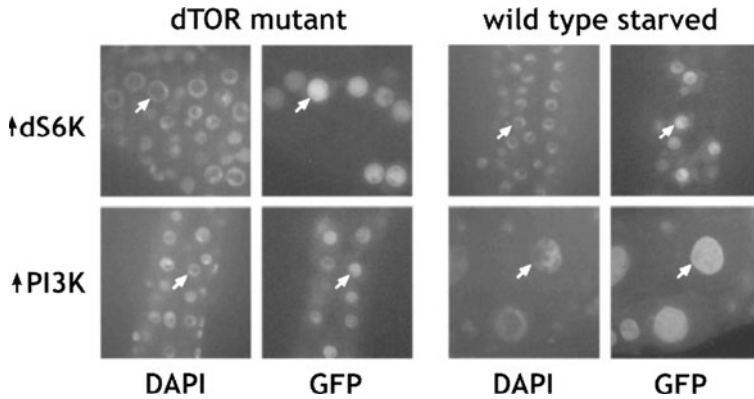
#### 4

#### Genetic Interactions with the Insulin/PI3K Pathway

The phenotypic similarities between *dTOR* and components of the insulin/PI3K signaling pathway are consistent with *dTOR* activity being required for productive signaling through this pathway. To test this model genetically, mosaic clones of cells doubly mutant for *dTOR* and *dPTEN*, a negative regulator of PI3K signaling, were examined. *dPTEN* mutant cells are increased in size and have an accelerated G<sub>1</sub> phase (Goberdhan et al. 1999; Huang et al. 1999; Gao et al. 2000). Both of these phenotypes are reversed in the doubly mutant cells, which are indistinguishable from cells lacking *dTOR* alone (Zhang et al. 2000). Thus, as in mammalian cells, *dTOR* is required for successful propagation of signals downstream of PI3K.

Numerous studies have implicated p70S6K as a key downstream target of both insulin and TOR-mediated signaling (Chung et al. 1992; Price et al. 1992; Brown et al. 1995; Weng et al. 1995). To test the extent to which *dTOR* signaling is channeled through p70S6K, the ability of transgene-driven overexpression of p70S6K to rescue *dTOR* mutant phenotypes was examined. Although the critical sites of p70S6K phosphorylation mediated by mTOR in vivo are not fully resolved, both Thr389 as well as proline-directed sites in the C-terminal pseudo-substrate domain show mTOR-dependent phosphorylation (Dennis et al. 1996; Burnett et al. 1998; Isotani et al. 1999). We find that overexpression of mutant versions of *dS6K* containing phospho-mimetic (acidic) residues, either at Thr398 (equivalent to Thr389 in human p70S6K) or in the pseudo-substrate domain, can provide substantial rescue of *dTOR* loss of function phenotypes. Constitutive, ubiquitous expression of either of these *dS6K* variants rescues hypomorphic *dTOR* mutants to viability, and rescues *dTOR* null mutants to later stages of development (Zhang et al. 2000; T.P. Neufeld, unpublished observations). The observation that mutation of either site is sufficient to partially bypass the requirement for *dTOR* suggests either that these sites are additive in their effect on *dS6K* activation, or that phosphorylation of one site acts as a priming step for phosphorylation of the other by a secondary kinase.





**Fig. 1** Rescue of growth inhibition by dS6K or PI3K overexpression. Photomicrographs of larval salivary glands in which the indicated genes are co-expressed with GFP in a subset of cells. Nuclear staining with DAPI allows the size of expressing and nonexpressing cells to be compared. All images shown are of the same magnification. *Top*: Overexpression of dS6K<sup>T398E</sup> restores growth in *dTOR* null mutant larvae (*left panels*), but not in wild-type larvae deprived of amino acids (*right panels*). *Bottom*: Overexpression of wild-type *Dp110* (PI3K catalytic subunit) increases the size of starved cells (*right*) but not of *dTOR* mutant cells (*left*)

We have extended these studies by testing the ability of various signaling components to activate cell growth and cell-cycle progression when overexpressed in individual cells within a *dTOR* null mutant background. Consistent with our previous results, overexpression of activated dS6K in single polyploid cells is able to partially override their growth arrest in *dTOR* null mutants animals (Fig. 1). In contrast, PI3K overexpression has no effect in the absence of dTOR, despite its potent capacity to induce cell growth in wild-type animals (Fig. 1, and see below). Together, these results provide strong genetic evidence that p70S6K is a major, biologically relevant TOR target *in vivo*. However, the greater phenotypic severity of *dTOR* mutants compared to *dS6K* mutants (Montagne et al. 1999; Oldham et al. 2000; Zhang et al. 2000), as well as the inability of dS6K overexpression to fully rescue *dTOR* null alleles, suggests that TOR also signals through other essential targets. Since it was recently reported that the growth effects of PI3K can be mediated independently of dS6K (Radimerski et al. 2002), our findings are consistent with a dS6K-independent function of dTOR being essential for PI3K-mediated growth.

## 5

***dTOR* Mutants Phenocopy the Effects of Starvation**

Withdrawal of amino acids from cells in culture causes dephosphorylation of TOR targets such as p70S6K and 4E-BP1 (Fox et al. 1998; Hara et al. 1998; Wang et al. 1998; Iiboshi et al. 1999; Kleijn and Proud 2000). Furthermore, a mutant form of p70S6K whose activity is resistant to rapamycin is also resistant to amino-acid depletion. These findings led to the proposal that mTOR activity is regulated in response to nutrient levels, consistent with models of TOR regulation in yeast. In *Drosophila*, nutrient deprivation results in a number of distinct phenotypes including developmental delay, cell-cycle arrest of specific cell types, changes in fat body morphology, sterility, and reduced body size (Robertson 1960; Bownes and Blair 1986; Britton and Edgar 1998; Britton et al. 2002). Interestingly, a number of these phenotypes are remarkably similar to those caused by mutations in *dTOR*. For example, either loss of *dTOR* function or deprivation of amino acids leads to a rapid cell-cycle arrest in polyploid (endoreplicative) tissues such as gut and salivary gland; in each case, the arrest can be bypassed by resupplying specific cell-cycle regulators such as cyclin E or E2F (Zhang et al. 2000). In contrast, diploid (mitotic) cells of the central nervous system and imaginal discs continue to cycle in starved or *dTOR* mutant animals. Another effect of starvation is to mobilize nutrient stores from larval fat body tissues, presumably to maintain viability of imaginal tissues when feeding conditions are poor. This can be visualized microscopically as a decrease in opacity of fat-body cells, and an increase in the size of lipid vesicles (Britton et al. 2002). Fat-body tissues from *dTOR* null mutant larvae are indistinguishable from those of starved animals, suggesting that similar cellular events are taking place (Zhang et al. 2000).

We recently isolated a number of new *dTOR* alleles containing point mutations in the N-terminal HEAT repeat region of the *dTOR* protein (C. Billington and T.P. Neufeld, unpublished data). These mutants behave as weak hypomorphs, and certain alleles and allelic combinations can survive to adulthood. Development in such cases is delayed, and surviving adults are reduced in size and are male- and female-sterile. Ovaries from sterile *dTOR* mutant females undergo an arrest of oogenesis at or prior to stage 8 (R. Scott and T.P. Neufeld, unpublished data), which was previously shown to contain a checkpoint for adequate nutrition (Bownes and Blair 1986). Thus, loss of *dTOR* results in a series of

cellular and physiological changes that closely resemble the effects of starvation.

## 6

### **Distinctions Between Starvation and *dTOR* Mutations**

Despite significant similarities, *dTOR* mutants and starved wild-type animals differ in important aspects of PI3K and dS6K signaling. As noted above, the arrested cell growth of *dTOR* mutants can be alleviated by overexpression of dS6K, but not of PI3K. In contrast, in larvae starved for amino acids, cell growth can be restored by overexpression of PI3K, but not dS6K (Fig. 1; see also Britton and Edgar 2002). Since dTOR activity is required for PI3K-mediated cell growth (Fig. 1), yet PI3K is able to promote growth under starvation conditions, it would follow that dTOR activity must be maintained during starvation. These results are at odds with observations that amino-acid insufficiency blocks insulin-mediated signaling to p70-S6K and 4E-BP1 in mammalian cultured cells (Hara et al. 1998; Iiboshi et al. 1999), findings which have been interpreted to indicate that mTOR activity may be regulated by nutrient levels. Although this discrepancy may be due in part to the greater reduction in nutrient levels achieved in culture than in vivo (due to the buffering effects of stored nutrients in the larva), the effective decrease generated by starvation in vivo is severe enough to fully arrest cell growth. Starvation has also been shown to rapidly reduce PIP(3) levels in *Drosophila* larval cell membranes, suggesting that PI3K activity is regulated in response to nutrition, perhaps via changes in levels of circulating insulin-like proteins (Britton et al. 2002). Therefore, data from these fly studies suggest that regulation of cell growth by nutrient levels in vivo are controlled to a greater degree by systemic, growth factor-mediated signaling than by direct cell-autonomous responses to nutrient-sensing pathways involving TOR.

## 7

### **Structural Insights from *dTOR* Mutations**

A number of point mutations in *dTOR* which lead to varying degrees of reduced function of the protein were recently isolated (C. Billington and T.P. Neufeld, unpublished data). These mutants can be divided into three distinct classes: class I consists of a series of weak hypomorphic (partial

loss of function) alleles which map to the N-terminal HEAT repeats of dTOR; class II is comprised of stronger hypomorphic mutations located in the kinase domain; and class III, which are null alleles corresponding to N-terminal stop codons or mutations in the kinase domain.

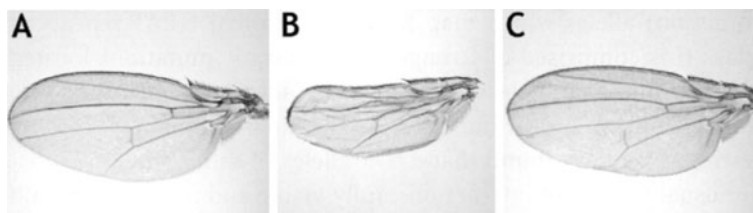
Interestingly, we have found that *dTOR* alleles of different classes display an unusual pattern of interactions: fully viable and fertile flies result from the trans-heterozygous combination of class I and class II alleles. In genetic terms, class I alleles, which disrupt the HEAT repeat region of dTOR, are able to complement class II alleles, which disrupt the kinase domain. Such intragenic interallelic complementation is uncommon in *Drosophila*, and is generally regarded as evidence of dimeric or multimeric assembly of gene products with two or more independent functional domains (Zabin and Villarejo 1975). Protein complexes containing a mixture of these defective dTOR proteins presumably remain active because the HEAT repeat function is supplied by the class II mutant dTOR protein, and the kinase function by the class I protein. In this regard, results from glycerol gradient centrifugation studies are consistent with dTOR assembling into a high-molecular-weight complex (M. Karter and T.P. Neufeld, unpublished data).

HEAT repeats have been shown to be important mediators of protein-protein interactions for a number of proteins (Kobe et al. 1999). In principle, both heterotypic interactions between TOR and its regulators and/or targets, as well as homotypic interactions between two or more TOR polypeptides could be mediated by the HEAT repeat domain of TOR. If the intragenic complementation described above indeed results from TOR multimerization, this would suggest that the major role of the HEAT region is for heterotypic binding, because three independent mutations in this region disrupt dTOR function yet apparently do not prevent dTOR self-association.

## 8

### Identification of TOR signaling Components via Genetic Interaction Screens

*Drosophila* has proven to be a useful system for isolating components of genetic pathways through the use of interaction screens. One strategy that has proven particularly successful has been to create genetic conditions that sensitize a given pathway to changes in activity or levels of pathway components. For example, a temperature-sensitive mutation in



**Fig. 2A–C** Genetic suppression of growth defects caused by dTOR overexpression. High levels of dTOR in the developing wing cause reduced growth and disorganization (B). Heterozygosity for a small chromosomal deficiency can significantly alleviate these effects (C). Genotypes: (A) wild type; (B) MS1096-Gal4/+, UAS-*dTOR*/+; (C) MS1096-GAL4/+, UAS-*dTOR*/Df(3L)Rdl-2

the Sevenless receptor tyrosine kinase has been used to generate threshold levels of signaling, such that heterozygous mutations that reduced the levels of downstream signaling factors could be identified as dominant enhancers or suppressors (Simon et al. 1991). Targeted overexpression of signaling proteins to specific tissues has also been successfully employed to generate sensitized conditions for such screens (Karim et al. 1996; Neufeld et al. 1998).

The TOR signaling pathway would appear to be ripe for such an approach. The mechanisms by which TOR is regulated, how it signals to downstream targets, and the identity of these targets are all unresolved. Moreover, the TOR protein has the potential for extensive protein-protein interactions via its HEAT repeats. Thus, it is likely that important TOR interactors remain to be identified.

We are pursuing two types of genetic screens aimed at identifying such interactors. First, screens are ongoing for mutations that can rescue the lethality or sterility of weak *dTOR* alleles. Such mutations would be expected to disrupt negative regulators of TOR signaling. This type of screen is particularly powerful in that positive selection strategies can be employed. A second type of screen underway in our laboratory utilizes targeted overexpression to generate a scoreable phenotype dependent on dTOR activity. We find that overexpression of either wild-type dTOR or of specific deletion mutants results in a strong suppression of growth (K. Hennig and T.P. Neufeld 2002). For example, overexpression of dTOR in the developing eye or wing causes a significant reduction in size of the resulting adult structures (Fig. 2). The severity of these phenotypes is sensitive to genetic background, and discrete chromosomal regions

have been identified which behave as genetic enhancers or suppressors (Fig. 2). Identification of the genes responsible for these interactions should add to our understanding of the complete repertoire of factors acting in the TOR signaling pathway.

## 9

### Conclusions

The identification of *dTOR* mutants allows the powerful tools of genetic analysis available in *Drosophila* to be applied to this signaling pathway. Many of the most interesting questions being addressed by studies of TOR in flies are similar to those being pursued in other systems:

1. What are the identity and nature of signals that regulate TOR activity? What is the change in cellular state brought about by nutrient deprivation which is recognized by TOR? To what extent do growth factor signaling pathways regulate TOR activity in vivo?
2. What are the effects of these pathways on TOR function? Changes in kinase activity, cellular localization, and protein-protein interactions are all potential points of modification.
3. Which cellular proteins interact physically with TOR? Does TOR form multiple types of complexes with distinct functions?
4. In addition to S6K, what are the physiologically relevant targets of TOR? Are such targets direct substrates of TOR, or does TOR signal by regulating phosphatase activities?

In addition to providing insights into TOR per se, these studies should also increase our understanding of how organisms coordinate genetically encoded developmental programs with the many environmental variables presented by nature.

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# Interplay Between Growth Factor and Nutrient Signaling: Lessons from *Drosophila* TOR

E. Hafen

Zoologisches Institut, Universität Zürich, Winterthurerstrasse 190,  
8057 Zürich, Switzerland  
E-mail: [hafen@zool.unizh.ch](mailto:hafen@zool.unizh.ch)

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**Abstract** During normal development, cellular and organismal growth is coordinately regulated. Each cell and each individual organ integrates information about nutrient availability, hormonal signals, and intrinsic growth programs. Describing the signaling pathways involved in these processes and how they are integrated is important to understand how growth is controlled during development and may also permit the development of means to curb uncontrolled growth in disease. In recent years, the biochemical analysis of cellular growth in cultured cells and the genetic dissection of growth control in model organisms has identified two conserved signaling pathways dedicated to cellular growth. The target of rapamycin (TOR) pathway regulates growth in response to nutrients, and the insulin/IGF pathways are involved in coordinating cellular growth in response to endocrine signals. This review discusses recent advances in the understanding of the interaction between these pathways, with a special focus on the contribution of the genetic analysis of these pathways in *Drosophila*.

## 1

**Introduction**

Applying genetics to developmental questions as it was pioneered by Nüsslein-Volhard and Wieschaus in the early 1980s led to significant progress in the understanding of the mechanisms by which pattern formation and cell fate specification are controlled during development of multicellular organisms. We have learned comparatively little about how the size of an organism, an organ, or an individual cells is controlled, although differences in size are among the most striking distinctions between species. Two reasons may explain why the genetic analysis of size control has lagged behind. First, body size varies gradually in response to environmental conditions such as nutrient availability and temperature. Therefore, mutations altering body size are more difficult to detect. Second, much of the research on cellular growth was concerned with the understanding of cell division and cell-cycle control. Although cell-cycle control is linked to cellular growth, it is not sufficient to regulate growth as defined by the increase in cell mass (Nurse 1975). In an elegant experiment, Neufeld and Edgar (Neufeld et al. 1998) demonstrated that this is also true for organ growth in *Drosophila*. They genetically marked clones of cells in the wing primordium of *Drosophila* and determined clone growth after a fixed time interval by measuring the area occupied by the clones in the epithelium. They also determined the size and number of the cells within the clones. When the cell cycle was accelerated in the clone by overexpressing dE2F, the overall growth of the clone did not change; the clone simply contained more but smaller cells. Conversely, slowing down the cell cycle by expressing *RBE*, the *Drosophila* Rb homolog, yielded fewer but bigger cells again without changing the overall area occupied by the clone. Therefore, in *Drosophila* epithelial cells, the cell cycle does not control cell growth.

Two different approaches lead to the discovery of genes involved in the control of cellular and organismal growth in *Drosophila*: the genetic characterization of genes encoding homologs of proteins involved in the regulation of cell growth in other organisms, and the unbiased search for mutations affecting organismal growth. The two approaches converged and demonstrate that target of rapamycin (TOR), ribosomal protein S6 kinase (S6K), and components of the insulin receptor/phosphatidylinositol-3-kinase (PI3K) pathway play a conserved role in the regulation of cellular and organismal growth (Kozma and Thomas 2002).

## 2

**Insulin Signaling Pathway and Growth Control in *Drosophila***

The insulin/IGF pathway plays a key role in regulating growth in invertebrates and vertebrates. In mammals, the primary role of insulin and insulin receptor is energy homeostasis by regulating blood glucose levels (Saltiel and Kahn 2001). However, mutations in the human insulin receptor gene also cause embryonic growth retardation (Takahashi et al. 1997; Taylor 1992). The primary growth regulatory function in mammals is mediated by growth hormone released by the pituitary. Growth hormone induces the expression of insulin-like growth factors 1 and 2 (IGF-1, 2) in peripheral tissues. IGF-1 and IGF-2 bind and activate the IGF receptor (IGFR1). Elimination of the function of IGF-1, IGF-2 or the corresponding receptor results in growth retardation and body-size reduction (Butler and Roith 2001). *Drosophila* has a single gene (*dInr*) encoding a homolog of the insulin/IGF receptor of vertebrates (Petruzzelli et al. 1986). As in vertebrates, lowering *dInr* function causes a reduction in overall body size (Chen et al. 1996), suggesting that the insulin/IGF receptor controls body growth in vertebrates and in invertebrates. Furthermore, insulin signaling in *Drosophila* is also involved in the regulation of energy stores and carbohydrate levels in the blood equivalent, the hemolymph. The genetic ablation of the neurosecretory cells that produce insulin-like peptides and release them into the hemolymph reduces body size and results in increased carbohydrate levels in the hemolymph (Ikeya et al. 2002; Rulifson et al. 2002). As in IRS-2 knock-out mice, lipid levels are increased in flies lacking the IRS homolog Chico (Böhni et al. 1999; Burks et al. 2000).

Biochemical analysis of insulin/IGF signaling in mammalian cells indicated the activation of two main signaling pathways in response to ligand binding. Activation of the Ras/MAP kinase pathway appears to be mediated by binding of the SH2 adaptor SHC to the activated IGF-receptor and has been postulated to control mitogenesis (Skolnik et al. 1993). Receptor activation also recruits adaptors of the insulin receptor substrate (IRS) family to the receptor. IRS is phosphorylated on multiple tyrosines that serve as docking sites for SH2-containing adaptors. Among those is the p85 subunit of the class 1A phosphatidylinositol-3-OH kinase (PI3K). Upon recruitment to the membrane, PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate on the 3' position of the inositol ring, generating phosphatidylinositol 3,4,5-trisphosphate (PIP3). Elevat-

ed concentrations of PIP3 at the plasma membrane recruit proteins containing pleckstrin-homology domains to the membrane, including phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB), also known as Akt (Vanhaesebroeck et al. 2001). Owing to a higher affinity of the PDK1 PH domain to PIP3 compared to the PKB PH domain, PDK1 is membrane-localized even in resting cells, whereas PKB is recruited to the membrane only upon insulin/IGF stimulation (Toker and Newton 2000). Membrane-localized Akt becomes further activated by direct phosphorylation by PDK1 and the as yet unidentified kinase PDK2 (Alessi et al. 1997; Stephens et al. 1998). Known targets of Akt include glycogen synthase kinase 3 (GSK3), the forkhead transcription factors of the daf-16/FKHR family and possibly target of rapamycin (mTOR) (Brazil and Hemmings 2001). In *Caenorhabditis elegans*, daf-16 is an important negative regulator of insulin signaling-controlled dauer formation, a developmental arrest program elicited by stress such as starvation (Ogg et al. 1997).

The PI3K pathway is kept in check by several negative regulators, among which the lipid phosphatase PTEN is the most prominent and best understood. PTEN removes the phosphate at the 3' position of the inositol ring of PIP3 and thus counteracts PI3K by reducing the concentration of PIP3 in the cell (Maehama and Dixon 1998). Its important role in growth control is underscored by the fact that in humans, the *Pten* gene is an important tumor suppressor gene mutated in almost as many tumors as p53 (Simpson and Parsons 2001).

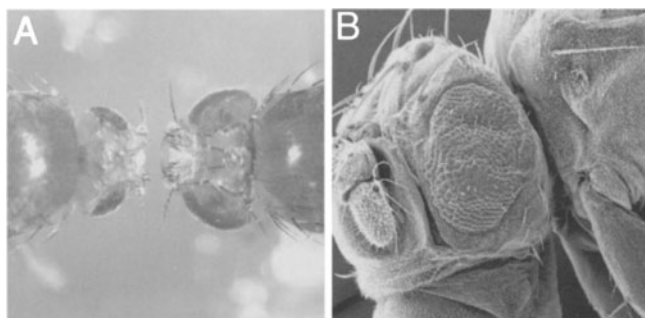
In *Drosophila*, the primary effector pathway downstream of the insulin receptor is the PI3K/AKT pathway. Although the Ras pathway promotes cell growth, cell survival, and differentiation, its activity appears to be controlled primarily by other receptors such as the EGF receptor (Diaz-Benjumea and Hafen 1994; Halfar et al. 2001; Prober and Edgar 2000). In contrast to the multiple functions of the Ras signaling pathway, the Inr-PI3K/Akt pathway appears to be dedicated to cell growth. Loss-of-function mutations in genes encoding Inr, Chico/IRS, dp110 PI3K or its SH2 adaptor dp60, and PKB cause very similar growth phenotypes (Böhni et al. 1999; Brogiolo et al. 2001; Verdu et al. 1999; Weinkove et al. 1999). Viable allelic combination in each of these genes produces flies of reduced body size caused by a reduction in cell size and in cell number. Mutant cell clones grow more slowly than neighboring cells. Interestingly, the slow growth is not due to an increase in apoptosis, as would be expected if this pathway played an important role in cell survival (Böhni

et al. 1999). The slower cell growth is accompanied by a proportional extension of all cell-cycle phases (Böhni et al. 1999). Conversely, removal of the negative regulator dPTEN stimulates growth by increasing cell size and cell number without influencing apoptosis (Gao et al. 2000). The concentration of PIP3 appears to be an important determinant of the rate of growth in each cell and may act as a second messenger for growth. Indeed, the lethality associated with a reduction in PIP3 levels in *dInr* mutants is rescued partially by a concomitant decrease in dPTEN activity (Oldham et al. 2002). The activation of dAkt and dPDK1 in response to the increased PIP3 levels is required for cell growth. Loss of dPDK1 function reduces cell growth and cell size and blocks S6K activity in *Drosophila* larvae, consistent with the role of mammalian PDK-1 in phosphorylating Akt and S6K (Radimerski et al. 2002b; Rintelen et al. 2001). Complete loss-of-function mutations in dAkt have a cell size and cell growth phenotype indistinguishable from mutations in *dInr* or *dp110-PI3K*. A partial loss-of-function mutation in *dAkt*, *dAkt<sup>3</sup>*, encodes a dAkt variant with an amino acid substitution in the PH domain, reducing its affinity for PIP3 (Stocker et al. 2002). Animals lacking dPTEN function possess increased PIP3 levels and die at the larval stage. Larvae that are homozygous for *dPTEN* and *dAkt<sup>3</sup>*, however, develop normally, and females are fertile (Stocker et al. 2002). Therefore, dAkt appears to be a key effector of the second messenger for growth, PIP3.

### 3

#### The TOR/S6K Pathway and Nutrient-Regulated Growth in *Drosophila*

The target of rapamycin (TOR) protein kinase controls cell growth by acting as a nutrient sensor in yeast and in mammalian cells (Dennis et al. 1999). In response to amino acids, TOR phosphorylates the elongation factor 4E binding protein (4EBP) and S6K (Gingras et al. 2001). S6K is phosphorylated and activated by TOR, but also requires phosphorylation by PDK1 and other kinases for full activity (Volarevic and Thomas 2001). S6K has been implicated in increasing the rate of translation of 5' terminal oligopyrimidine tract (TOP) mRNAs that encode components of the translational machinery including ribosomal proteins (Fumagalli and Thomas 2000). 4EBP blocks translation initiation by binding to the CAP binding protein eIF4E. Upon TOR-mediated phosphorylation, 4EBP dissociates from eIF4E, thus permitting initiation of translation (Gingras et al. 2001).



**Fig. 1A, B** Loss of dTOR function in the head progenitor cells reduces cell growth but does not affect differentiation. **A** Comparison of a wild-type fly (*right*) with a genetically mosaic fly (*left*) homozygous for a dTOR loss-of-function mutations in the head tissue and heterozygous in the body. The genotype of the fly *y w eyFLP; FRT40 dTOR<sup>22.1</sup>/FRT40 P(w<sup>+</sup>) l(2)3.1*. **B** Scanning electron micrograph of *dTOR* mutant head. Note that the absence of dTOR function does not block differentiation of head tissue but reduces growth of the tissue

Genetic analysis of TOR function in *Drosophila* provided evidence that TOR acts as a nutrient sensor in multicellular organisms. Mutations in *dTOR* have been identified in two ways. Zhang et al. (2000) identified the *dTOR* gene by homology search and subsequently isolated deletion mutants by mobilizing a nearby transposable element. Oldham et al. (2000) identified alleles of variable strength in a genome-wide search for recessive mutations affecting cell growth. Using a tissue-specific recombination system (Newsome et al. 2000), mosaic flies were generated that contain head tissue homozygous for randomly induced mutations on a specific chromosome arm, whereas cells in the body and the germline are heterozygous for the same mutations. Mutations in genes that function in cell growth but not differentiation are identified as flies with small heads in the case of genes with a growth-promoting function, and flies with large heads in the case of genes with a growth-inhibiting function (tumor suppressor genes). In this way, recessive mutations with a growth phenotype can be identified in individual flies in the F1 generation. This efficient screen has identified mutations in all known components of the insulin receptor pathway and several novel loci that are currently being characterized (H. Stocker, S. Oldham, S. Breuer, and E. Hafen, unpublished results). Mosaic flies homozygous for *dTOR* mutations

in the head possess small but normally differentiated heads (Fig. 1) (Oldham et al. 2000).

The phenotype of *dTOR* mutants is similar yet distinct from that of mutations in genes encoding dInr, dp110-PI3K, Chico/IRS, or dAkt. As in the case of dInr pathway mutations, *dTOR* mutant cells are able to differentiate, but cell size is severely affected. Homozygous *dTOR* mutant animals fail to pupariate but remain at the larval stage for an extended period of time before they die. However, although a reduction in insulin signaling causes a proportional reduction of growth of all tissue, reduction in dTOR function causes a preferential reduction of the larval endoreplicating tissue, a reduction in the size of the nucleoli, and an increased accumulation of lipid vesicle in the larval fat body (Oldham et al. 2000; Zhang et al. 2000). These phenotypes resemble those of wild-type larvae starved for amino acids (Britton and Edgar 1998) and are thus consistent with a role of dTOR in sensing nutrients.

The genetic and biochemical analysis of dS6K as a target of dTOR supports the nutrient-sensing role of this pathway. In contrast to the lethality associated with *dTOR* mutants, animals homozygous for *dS6K* null alleles develop to the adult stage, albeit with a substantial developmental delay (Montagne et al. 1999). The more severe lethal phenotype caused by the absence of dTOR suggests that dTOR exerts its effect not only through dS6K but also through other targets. Furthermore, *dTOR* mutants are only partially rescued by overexpression of dS6K variants (Zang et al. 2000; J. Montagne and G. Thomas, personal communication). Interestingly, 4EBP, the other well-established target of TOR, does not seem to be essential for normal growth regulation. Loss-of-function mutations in the single *4EBP* gene in *Drosophila* are viable with no apparent growth deficit (Miron et al. 2001). In contrast, *dS6K* mutant flies are semi-lethal and slightly reduced in size (Montagne et al. 1999). The reduction in body size is due to a reduction in cell size only. dS6K activity is dependent on dTOR and nutrient availability. In protein extracts for *dTOR* mutant larvae or larvae that have been starved or treated with rapamycin, dS6K activity is severely reduced (Radimerski et al. 2002a).

#### 4

### **TSC1/2 Act as Negative Regulators of TOR**

The tuberous sclerosis complex (TSC) complex is a dominant disorder occurring in about 1/6,000 human births and is characterized by the



presence of benign tumors with large cells (hamartomas) in many organs including brain, skin, and kidney (Cheadle et al. 2000). Heritable forms of TSC are associated with mutations at two loci, *TSC1* and *TSC2*. *TSC1*, also called hamartin, encodes a 1,164 amino acid protein containing two putative coiled-coil domains. *TSC2* encodes tuberin, a 1,807 amino acid protein with coiled-coil domains and a short domain that shares homology with the GTPase activating protein (GAP) of the Rap GTPase (Cheadle et al. 2000). Hamartin and tuberin have been shown to interact physically. From these human genetic and biochemical studies, little was known about how these proteins function. The genetic characterization of the TSC complex in *Drosophila* demonstrated that it negatively regulates growth through the dTOR nutrient-sensing pathway. Mutations in the genes coding for the *Drosophila* dTSC1 and dTSC2 have been identified in screens for mutations promoting cell growth. Loss of either dTSC1 or dTSC2 function promotes cellular growth and leads to a significant increase in cell size (Ito et al. 2000; Gao and Pan 2001; Potter et al. 2001; Tapon et al. 2001). Genetic interactions between dTSC mutations, components of the insulin signaling pathway, and dS6K place TSC function between dAkt and dS6K. Partial loss of dTSC function is sufficient to rescue the lethality associated with loss-of-function mutations in *dInr* (Gao and Pan 2001). Furthermore, complete loss of dTSC1 or dTSC2 function fully restores the growth and cell-size reduction of cells homozygous for *dAkt* and *dInr* mutations but not of *dS6K* mutants (Gao and Pan 2001; Potter et al. 2001). These results would be consistent with a model in which the dTSC complex acts in a linear pathway downstream of dAkt. However, cells doubly mutant for *dPTEN* and *dTSC1* are almost twice the size of cell homozygous for either of the two tumor suppressor mutant alleles (Gao and Pan 2001). Thus it appears that dTSC and dPTEN act in two parallel pathways.

The close interaction between TSC tumor suppressor complex and TOR is further supported by recent experiments carried out in *Drosophila* and in mammalian cells. *dTOR* mutations completely block the cell overgrowth phenotype in *TSC* mutant clones (Gao et al. 2002). Furthermore, lethality associated with loss of dTSC function is rescued by partially reducing dTOR and dS6K function in *dTOR*, *dS6K* double heterozygotes (Radimerski et al. 2002a). Consistent with the genetic studies, RNAi-mediated reduction of *TSC2* function in mammalian cells and in *Drosophila* S2 cells increases S6K activity. This increase is blocked by inhibiting TOR function by rapamycin (Gao et al. 2002; Inoki et al. 2002;

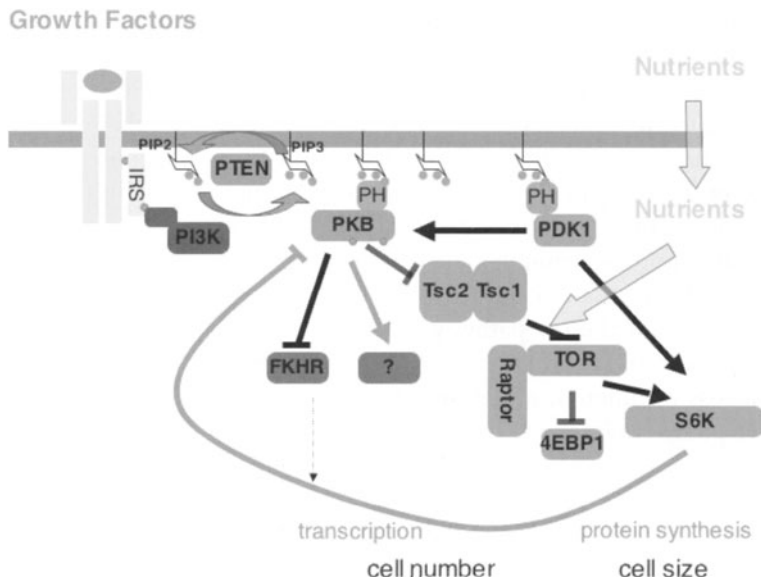
Radimerski et al. 2002a). Co-immunoprecipitation studies in S2 cells further suggest that dTSC2 associates with dTOR (Gao et al. 2002). This association appears to be important for the downregulation of S6K activity in response to starvation. Whereas withdrawal of amino acids from the medium results in a drop of S6K activity within 15 min, a concomitant decrease in dTSC2 function is sufficient to block this decrease (Gao et al. 2002). From these genetic and biochemical data, it is evident that the TSC tumor suppressor complex negatively regulates TOR and S6K activity and that this regulation appears to be independent of PTEN. Yet, local hyperactivation of the Inr pathway in the fat body results in increased nutrient uptake and lipid storage, a phenotype strikingly opposite that of *dTOR* loss-of-function mutations (Britton et al. 2002). How then are these two pathways integrated?

## 5

### Interaction Between the TSC-TOR and Akt-PTEN Pathways

Recent reports indicate that TSC2 is directly phosphorylated by Akt (Inoki et al. 2002; Manning et al. 2002; Potter et al. 2002). Akt-mediated phosphorylation of TSC2 targets it for degradation, thus resulting in an increase in S6K activity. dAkt also phosphorylates dTSC2 at two sites that are conserved in the human TSC2 in S2 cells, and weakens the interaction between dTSC1 and dTSC2. Whereas the suppression of the dAkt-induced overgrowth phenotype in the developing eye requires the co-expression of dTSC1 and dTSC2, expression of a nonphosphorylatable form of TSC2 alone is sufficient to suppress the phenotype (Potter et al. 2002).

The ability of Akt to regulate the stability of the TSC complex by phosphorylation induced degradation of TSC2, and thus activation of TOR-dependent S6K activity provides a link between S6K activation by insulin and amino acids (Fig. 2). However, there is some evidence that is inconsistent with this being the main point of integration. It must be kept in mind that these experiments were performed in cell culture or involve overexpression in *Drosophila* and thus may not reflect the activity levels of these pathways in normal cells. Radimerski et al. (2002b) examined the activity of dS6K in protein extracts of wild-type and mutant larvae under normal and starvation conditions. dS6K activity in vivo is reduced by rapamycin treatment, amino acid starvation, and in a *dTOR* mutant background. Surprisingly, however, dS6K activity is unchanged



**Fig. 2** The insulin signaling network. The signaling pathways that are activated by insulin-like growth factors and nutrients and that control cell and organ growth are shown. *Black connectors* indicate direct physical interactions and/or phosphorylation or dephosphorylation steps. *Gray connectors* indicate interactions that have been established genetically but for which the molecular mechanisms have not yet been elucidated

in a *chico*/IRS mutant background. Thus, conditions that lower insulin signaling in a manner that results in a severe growth retardation—*chico* mutant flies are approximately half the weight of normal flies—do not affect dS6K activity. Even more surprisingly, *Drosophila* larvae homozygous for *dAkt* loss-of-function mutations have normal dS6K levels. If dAkt plays a key role in regulating dS6K activity by phosphorylating and destabilizing dTSC2, S6K activity should drop in dAkt mutants (Radimerski et al. 2002a). In *dAkt* mutant cells, dPDK1 should be activated normally and may thus permit sufficient activation of S6K. Alternatively, it is possible that another kinase is involved in phosphorylating dTSC2.

In vivo, dS6K activity may be regulated at two different levels. Under suboptimal growth conditions that permit flies to develop to adults but with severely reduced body weight—this situation is mimicked by *chico* mutants—dp110-PI3K activity is low and produces low levels of PIP3.

These low levels of PIP3 are sufficient to recruit dPDK1 (dPDK1 activity towards S6K activity is PIP3-independent) but not dAkt to the membrane and activate it. The presence of a low amount of amino acids also stimulates dTOR in the presence of dTSC to phosphorylate S6K. Together with the input from PDK1 S6K is active. The growth deficit under these conditions would be largely due to low activity of dAkt and its yet unknown downstream effectors. Under optimal growth conditions, increased levels of insulin-like peptides in the hemolymph stimulate the insulin pathway generating high levels of PIP3 and thus fully activating dAkt that, under these conditions, phosphorylates dTSC2 and thus increases dTOR activity leading to a maximal stimulation of dS6K activity. Consistent with this hypothesis is the fact that dS6K activity in S2 cells depleted for dTSC2 by RNAi can no longer be increased by insulin treatment (Radimerski et al. 2002a). The test of the role of dAkt in regulating dTSC2 stability in vivo awaits the rescue of *dTSC2* null mutants with a transgene that encodes a dTSC2 variant that cannot be phosphorylated by dAkt.

In *Drosophila*, so far three links between nutrient availability and insulin signaling are evident. First, of the three genes coding for insulin-like peptides (*dilp2*, 3, and 5) that are expressed in the secretory cells in the brain, the expression of *dilp3* and *dilp5* is reduced under starvation conditions (Ikeya et al. 2002). Thus starvation results in a reduction in insulin-like growth factors in the hemolymph. As has been shown for other insects (Masumura et al. 1997), starvation probably also regulates the release of insulin from secretory vesicles analogous to the regulation of insulin release from beta-cells in the pancreas in mammals. Second, Akt may regulate the S6K/Tor pathway by controlling the stability of the TSC complex. Third, the dTOR/dS6K pathway also acts negatively on dAkt activity. The activity of dAkt is increased in protein extracts from dTOR or dS6K larvae but decreased in the absence of dTSC function (Radimerski et al. 2002a, b). The mechanism of the negative feedback loop is unknown.

## 6

### Concluding Remarks and Open Questions

The insulin and TOR pathways form a signaling network that integrates information about nutrient availability and an intrinsic developmental program. Although significant progress has been made in identifying

components of this network and in understanding their interaction, there are still a number of important questions to which genetic analysis in model organisms may provide answers. (1) What are the downstream effectors of Akt, TOR, and S6K that promote cell growth? Mutations in the known established targets such as GSK3 and dFKHR in the case of dAkt may help to answer these questions. In addition, the continued search for new genes involved in growth control is important to identify new functionally relevant elements in the network. (2) How does pattern formation during development interface with cell growth? Morphogen gradients play an important role in generating the final form by local regulation of growth (Martin-Castellanos and Edgar 2002). How do these developmental signals interface with the growth regulatory network described here? (3) What are the genes involved in the uncontrolled growth of tumor cells lacking different tumor suppressor genes? These may be important targets for therapeutic invention.

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# mTOR Signaling to Translation

A.-C. Gingras<sup>1, 2</sup> · B. Raught<sup>1, 2</sup> · N. Sonenberg<sup>1</sup>

<sup>1</sup> Department of Biochemistry, McGill Cancer Centre, McGill University,  
3655 Promenade Sir-William-Osler, Montréal, Québec, H3G 1Y6, Canada  
*E-mail: nsonen@med.mcgill.ca*

<sup>2</sup> Institute for Systems Biology, 1414 North 34th Street, Seattle, WA 98103, USA

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**Abstract** Over the past few years, the target of rapamycin (TOR) pathway has been implicated in the control of translation, both in yeast and in higher eukaryotes. In this review, we provide an overview of translation in eukaryotes, and discuss the mechanisms and advantages of the regulation of translation. We then describe how the TOR pathway can modulate translation in yeast and in mammals, through the modulation of the phosphorylation of key translation components, and the regulation of the abundance of ribosomes and translation factors.

## 1

**Introduction**

Regulation of translation in eukaryotes plays a critical role in the control of cell growth and proliferation. In higher eukaryotes, translational control has also been implicated in a wide range of other physiological phenomena, such as development, memory formation, and apoptosis. One of the main advantages of regulating translation (as opposed to transcription, for example) is that the response to a given stimuli can be very rapid and energy efficient; no mRNA synthesis, processing, or transport is required.

How information from extracellular stimuli is relayed to the translation machinery has been the focus of a wealth of research spanning many years. In the past several years, however, it has become apparent that the abundance and/or activity of multiple molecules required for translation (e.g., ribosomal RNA, ribosomal proteins, and translation initiation and elongation factors) are regulated, at least in part, via the target of rapamycin (TOR) pathway. Here, we provide a brief overview of translation in eukaryotes, followed by a discussion of the role of the TOR pathway in translational control.

## 2

**General Mechanism of Translation**

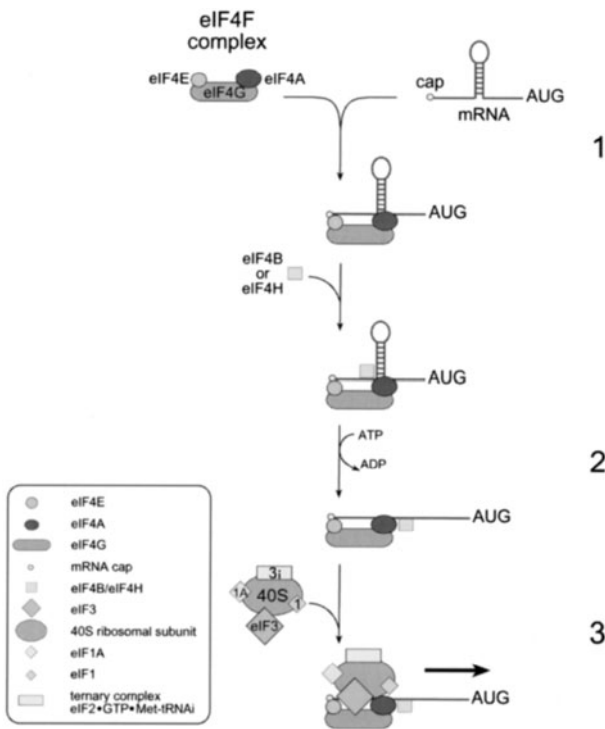
Translation is divided into three phases: initiation, elongation, and termination. Initiation consists of the recruitment of a ribosome to an mRNA, and its positioning at an initiation codon (usually AUG, but other codons are occasionally utilized). Elongation is the “translation” phase proper, in which aminoacyl-tRNAs are bound by the ribosome and joined to the carboxy-terminus of the nascent polypeptide chain. Termination, prompted by the recognition of a stop codon, results in the dissociation of ribosomes from mRNA, and the release of a mature polypeptide.

Translation initiation is a complex, multistep process, requiring numerous translation initiation factors (about 30 polypeptides; reviewed in Dever 2002; Hershey and Miyamoto 2000). Initiation commences with the association of the 40S ribosomal subunit with an array of translation initiation factors (eIF1, eIF1A, eIF3, and eIF5). The initiation factor eIF2 forms a stable ternary complex with the methionyl initiator tRNA (Met-

tRNA<sub>i</sub><sup>Met</sup>) and GTP, and delivers the Met-tRNA<sub>i</sub><sup>Met</sup> to the ribosomal P site. Once the eIF2\_GTP\_Met-tRNA<sub>i</sub><sup>Met</sup> ternary complex is assembled onto the 40S subunit, it is competent to bind to mRNA. However, the ribosome itself is unable to locate and bind to the 5' region of mRNA, a critical step for the translation of most cellular mRNAs. Instead, the ribosome must be recruited to the mRNA via the eukaryotic translation initiation factor 4 (eIF4) proteins.

Eukaryotic mRNAs possess at their extreme 5' terminus the "cap" structure, m<sup>7</sup>GpppX (where m is a methyl group, and X any nucleotide). The cap structure is specifically recognized by a cap binding protein, the initiation factor eIF4E (see Fig. 1). eIF4E, together with eIF4A (an RNA helicase) and eIF4G (a large scaffolding molecule) form a trimeric complex termed eIF4F. In conjunction with an RNA-binding protein, eIF4B (and likely an eIF4B homolog, eIF4H), eIF4F is thought to catalyze the unwinding of inhibitory secondary structure in the mRNA 5'UTR (Hershey and Miyamoto 2000). In addition, eIF4G binds to the ribosome-associated factor eIF3. Once bound near the mRNA 5'end, the 40S ribosomal subunit, plus the cohort of associated factors, is thought to scan in a 5' to 3' direction, in an energy-dependent manner, until it encounters a start codon in a particular sequence context (Hershey and Miyamoto 2000). Proper positioning of the ribosome at a start codon is dependent on base pairing between the AUG and the tRNA<sub>i</sub><sup>Met</sup> anticodon, and requires the initiation factors eIF1, eIF1A, eIF2, and eIF5. Following the recognition of an initiation codon, the eIF2-bound GTP is hydrolyzed to GDP (in a reaction catalyzed by eIF5), and most of the factors dissociate from the 40S subunit. At this point, when the tRNA<sub>i</sub><sup>Met</sup> is positioned in the ribosomal P site and establishes base-pairing with the start codon, the 60S subunit joins the mRNA-associated 40S subunit, in a step catalyzed by the GTP-bound factor eIF5B. Upon joining of the two ribosomal subunits to form an 80S ribosome, eIF5B hydrolyzes GTP to GDP, and is released from the ribosome. Only at this stage can peptide elongation begin.

The speed and accuracy of elongation are regulated by the translation elongation factors eEF1A, eEF1B, and eEF2, which catalyze this process in an energy-dependent manner (provided via GTP hydrolysis). The process of elongation, and its regulation, is discussed in depth in (Merrick and Nyborg 2000; Proud 2000). When a stop codon is located in the ribosomal A site, polypeptide chain release factors (eRFs) are recruited to the ribosome to catalyze the hydrolysis of the ester bond between the



**Fig. 1** Ribosome recruitment. Through eIF4E, eIF4F binds to the mRNA 5' cap structure (1). eIF4A, along with the RNA-binding protein eIF4B (and likely eIF4H), melts 5'UTR secondary structure to provide a single-stranded ribosome landing pad (2). In a separate step (not shown here), the 40S ribosomal subunit is rendered competent for translation via the binding of eIF1, eIF1A, eIF3, and the ternary complex eIF2\_GTP\_Met-tRNA<sub>i</sub>. The 40S ribosomal subunit is recruited to the mRNA, through an interaction between eIF3 and eIF4G (3), then scans the 5'UTR in a 5'-3' direction until it encounters an initiation codon

final amino acid of the polypeptide chain and the tRNA, resulting in the release of the mature polypeptide chain. Following this event, termination factors are also released, and the ribosomal subunits are recycled for another round of initiation, via a mechanism which remains ill-defined in eukaryotes (reviewed in Welsh et al. 2000).

While the initiation mechanism described above (so-called cap-dependent translation) is utilized by most cellular mRNAs, alternative initiation mechanisms also exist (Jackson 2000). Perhaps the best charac-

terized example of cap-independent translation is that mediated by an internal ribosome entry site (IRES; e.g., Jang et al. 1988; Pelletier and Sonenberg 1988). IRESes are found in the 5'UTRs of many viral RNAs (the classical example being picornavirus RNA), as well as in several cellular mRNAs (reviewed in Hellen and Sarnow 2001). Picornavirus IRESes are highly structured, and would be expected to be poorly translated according to the scanning model of cap-dependent translation. However, these structures confer very efficient translation via direct recruitment of ribosomes, in a manner independent of the cap structure and eIF4E. Consistent with this, IRES-driven translation proceeds efficiently under conditions in which the abundance and/or activity of eIF4E is reduced, such as during mitosis (Bonneau and Sonenberg 1987; Pyronnet et al. 1998). There are several cellular mRNAs whose mode of translation appears to toggle between a cap-dependent and an IRES-driven mode, depending, for example, on the phase of the cell cycle, the availability of nutrients, or the induction of an apoptosis program (reviewed in Hellen and Sarnow 2001; Holcik et al. 2000).

### 3

#### **Regulation of Translation**

Although each of the translation steps can in principle be subject to regulation, the limiting step under most circumstances appears to be initiation. Besides being more energy-efficient than regulating protein synthesis during the elongation or termination phases, it may be important to control translation at the initiation phase to prevent the production of potentially deleterious truncated peptides. However, there are instances in which the other phases, especially elongation, become limiting (reviewed in Mathews et al. 2000; Proud 2000).

It would be logical to assume that an increase in the activity of the general translation machinery would lead to a global, proportionate increase in the translation of all mRNAs. However, experimental data have not borne out this type of model. Instead, an abundance of evidence suggests that modulation of general translation factor activity disproportionately affects the translation of a specific subpopulation of mRNAs (for an early review, see Lodish 1976). For example, treatment of mammalian cells with hormones or growth factors leads to a relatively small (usually up to ~twofold) increase in global protein synthesis (e.g., Manzella et al. 1991). By contrast, a dramatic increase is observed in the

translation rates of some mRNAs: e.g., the translation rate of the ornithine decarboxylase (ODC) mRNA increases 30-fold in response to insulin treatment (Manzella et al. 1991). What might be the basis for this discrimination? An attractive model originally proposed by Lodish more than a quarter of a century ago suggested that mRNAs are translated in a competitive manner, with some mRNAs being “weak” or poorly translated under most conditions, while other mRNAs are naturally “strong” and normally efficiently translated (Lodish 1974). If the activity of the translation machinery increases, the “strong” mRNAs continue to be translated very efficiently, but the “weak” mRNAs are also now translated efficiently, due to a diminished competition for the translation apparatus. In this way, changes in the activity of the general translation machinery can effect a dramatic modulation of the translation rates of some mRNAs, while only marginally affecting the translation rates of others.

What might determine the relative “strength” of a specific mRNA? In fact, mRNAs possess a wide variety of *cis*-acting elements that can profoundly influence translation efficiency. These structural elements may function in combination with the general translation machinery, or with specific *trans*-acting factors (reviewed in Mathews et al. 2000). *Cis*-acting elements acting in combination with general translation components include the sequence flanking the initiation codon (start site context), the presence of secondary structure in the 5' UTR (see below), upstream AUG triplets in the 5'UTR, upstream open reading frames, and various elements in the coding sequence. *Cis*-acting elements acting with specific *trans*-acting factors may function in either a positive or a negative manner, and generally act by providing binding sites for *trans*-acting factors.

One of the best studied classes of *cis*-acting elements is 5'UTR secondary structure, commonly found in mRNAs coding for oncogenes and growth-promoting proteins (Kozak 1991). This secondary structure is thought to be melted by eIF4F (in conjunction with eIF4B) to allow the ribosome access to the mRNA 5' UTR (e.g., Rozen et al. 1990). When eIF4F activity is low, translation of “weak” mRNAs (those possessing extensive secondary structure) is impeded, an effect that is relieved when eIF4F is more active (Sonnenberg 1993). Consistent with these findings, overexpression or mutation of eIF4B, or of the individual eIF4F components, affects the translation of weak mRNAs to a greater extent than strong mRNAs (e.g., Altmann et al. 1995; Koromilas et al. 1992; Svitkin et al. 2001).

Several specific 5'- or 3'-UTR sequence elements have also been demonstrated to modulate translation efficiency. For example, the 5' terminal oligopyrimidine tract (5'TOP) is a cap-proximal structural element (consisting of an uninterrupted stretch of 4–14 pyrimidines) that confers stringent regulation of translation (reviewed in Fumagalli and Thomas 2000; Meyuhas and Hornstein 2000). Under conditions of nutrient or growth factor deprivation, or following the initiation of a differentiation program, the translation of 5'TOP-containing mRNAs is potently repressed. Stimulation via nutrients, hormones, cytokines, or growth factors, for example, overcomes the translational repression. mRNAs coding for components of the protein synthesis machinery constitute the large majority of this class of mRNAs. The ~40 vertebrate ribosomal proteins analyzed thus far all bear 5'TOP elements, and are translationally controlled in a growth-dependent manner (Meyuhas and Hornstein 2000). Additional translation machinery components possess 5'TOPs, including elongation factors (eEF1A, eEF1B, eEF2), and the poly(A) binding protein (PABP), as well as proteins involved in ribosome biosynthesis and assembly (Meyuhas and Hornstein 2000). Whether the translational regulation of the 5'TOP mRNAs is dependent on a specific *trans*-acting factor, or on modification of a component of the general translation machinery has not been determined. Several *trans*-acting factors have been proposed to modulate 5'TOP activity: two RNA-binding proteins from *Xenopus*, the La autoantigen and CNBP (cellular nucleic-acid binding protein), as well as a murine lymphocyte protein, p56<sup>L32</sup>, were shown to interact with the 5'TOP and adjacent sequences (reviewed in Meyuhas and Hornstein 2000). However, the mechanism of their effects on ribosome recruitment, and on the regulation of 5'TOP activity, remains unclear.

The fraction of mRNAs subject to translational regulation remains an open question, and is most likely dependent on cell type, cell stage, and the particular stimulatory or inhibitory treatment applied. In recent years, combining polysome fractionation with cDNA array technologies has provided estimates of the number and identity of differentially-translated mRNAs in several different cell types (e.g., Carter et al. 2000; Zong et al. 1999). When applied to a sucrose density gradient, mRNAs sediment according to the number of associated ribosomes. In general, mRNAs in the process of being efficiently translated are associated with many ribosomes; these mRNA-multiribosome structures are termed polysomes, and sediment toward the bottom of the gradient. In contrast,

mRNAs that are poorly translated (or untranslated) are found in the messenger ribonucleoprotein particles or are associated with few ribosomes, and thereby fractionate toward the top of the density gradient. The gradient may be fractionated, and the identity of the mRNAs present in each fraction determined using DNA chip or array technologies (for a review of this technique, refer to Carter et al. 2000). For example, Zong et al. reported that upon serum-stimulation of fibroblasts, ~1% of mRNA transcripts (of 1,200 genes screened) shift to polysomes (Zong et al. 1999), while Mikulits et al. reported the recruitment of nearly 10% of mRNA transcripts into polysomes following the activation of resting T cells (472 genes screened; Mikulits et al. 2000). This technique can also be used to investigate the effect of individual *trans*-acting factors on the translation of specific mRNAs. For example, Brown et al. analyzed the consequences of disrupting FMR, an RNA-binding protein defective in cells harboring a fragile X mutation. In mutant cells, the polysome profiles for ~2% of the total mRNA transcripts (of 11,000 detected), and 50% of known FMR targets (14/28), were altered as compared to normal cells (Brown et al. 2001). As the use of these techniques expands, our understanding of the nature of translationally regulated mRNAs will improve accordingly.

## 4

### Regulation of Translation by Rapamycin

#### 4.1

##### Overview of the Rapamycin-Sensitive Signaling Pathway

Rapamycin is a lipophilic compound originally isolated from a micro-organism indigenous to Easter Island (a.k.a. Rapa nui), and first characterized for its potent antifungal properties (Vezina et al. 1975). In the early 1990s, it was discovered that the effects of rapamycin are not limited to fungi: it was also found to inhibit mammalian T cell activation and proliferation, and to act as a potent immunosuppressant (Abraham and Wiederrecht 1996). In fact, rapamycin derivatives are now used in the clinic, in combination with other immunosuppressants, to prevent graft rejection (Kahan 2001; MacDonald 2001). Later studies demonstrated that the proliferation of many other mammalian cell types is also inhibited by rapamycin. Since rapamycin also inhibits tumor growth (possibly by eliciting apoptosis in tumor cells), and impairs angiogenesis



(Guba et al. 2002), it is a promising anticancer agent, and is now undergoing clinical trials for this application (Huang and Houghton 2002).

The mode of action of rapamycin is conserved from yeast to mammals. Upon entrance into the cell, rapamycin binds to one or more of a group of immunophilins termed the FK-binding proteins (or FKBP, so-called because of their identification as binding partners for FK506, a compound structurally related to rapamycin; reviewed in Abraham and Wiederrecht 1996; Gingras et al. 1998). In mammalian cells, the primary rapamycin-binding protein is FKBP12, a 12-kDa member of this family. The FKBP is a peptidyl-prolyl *trans*-isomerase, which presumably assist in protein folding (Gingras et al. 1998). While rapamycin can inhibit FKBP isomerase activity (at least in vitro), inhibition of FKBP activity does not mimic the effects of rapamycin treatment: in yeast, simultaneous deletion of all four FKBP genes does not recapitulate the rapamycin phenotype. Subsequent investigations revealed, instead, that an FKBP-rapamycin gain-of-function complex binds to and disrupts the function of the target of rapamycin (TOR) proteins (Abraham and Wiederrecht 1996; Thomas and Hall 1997).

Two TOR proteins (TOR1 and TOR2) are present in *S. cerevisiae*, but only one TOR family member is present in metazoans, including *D. melanogaster* (dTOR) and mammals (mTOR, also referred to as FRAP or RAFT). The TOR proteins are highly evolutionarily conserved, sharing a striking degree of homology and identical structural organization. All TOR C-termini contain a domain with homology to phosphoinositide kinase-related kinases (PIKKs), most closely resembling that found in ATM (Ataxia telangiectasia mutated), ATR (ATM and Rad3 related) and DNA-PK (DNA-activated protein kinase, catalytic subunit; Keith and Schreiber 1995). While the PIKKs were first described as possessing homology to lipid kinases, they function instead as protein kinases (Hunter 1995). Immediately amino-terminal to the kinase domain lies the FKBP-rapamycin binding (FRB) domain. Mutation of a key serine residue in the FRB domain (Ser2035 in human TOR) to bulkier residues (e.g., threonine, glutamic acid, or isoleucine) inhibits rapamycin-FKBP binding, and engenders rapamycin resistance (Abraham and Wiederrecht 1996). This finding has allowed researchers to demonstrate the involvement of TOR proteins in all rapamycin effects reported to date. The TOR protein N-termini contain several "HEAT" repeats (named for Huntingtin, elongation factor 3, the adaptor (A) subunit of PP2A, and TOR1, the first proteins found to contain such repeats), a motif found in

several molecular scaffolding proteins, and likely to mediate protein-protein interactions (Andrade et al. 1995; Groves and Barford 1999). Indeed, the HEAT domain of TOR has been found to bind to at least two partners: gephyrin (a tubulin-binding protein involved in postsynaptic clustering of neuronal glycine receptors; Sabatini et al. 1999), and a novel protein termed RAPTOR or COG (Regulatory Associated Protein of mTOR; Control Of Growth, see the chapter by Sabatini and the chapter by Hall, this volume). The TOR proteins also possess additional critical, but less well-characterized sequence motifs. There are two stretches of homology with the other PIKKs (and which are absent in PI3 and PI4 lipid kinases): the FAT (FRAP, ATM, TRAPP) and FATC (FAT, carboxy terminal) domains, located in the midregion (amino acids 1,382–1,982 in human mTOR) and at the extreme 3' end of the TOR proteins, respectively (Bosotti et al. 2000). The FAT and FATC domains, always found in combination, are presumed to be involved in intramolecular interactions (Bosotti et al. 2000).

The TOR proteins, and the consequences of rapamycin treatment on TOR activity, are discussed in detail in accompanying chapters (see the chapters by Lawrence, Hall, Sabatini, and Thomas, this volume). Of particular importance to the present discussion, rapamycin appears to inhibit downstream TOR signaling not solely by inactivation of TOR kinase activity, but, at least in part, via the activation of one or more TOR-regulated protein phosphatases. In yeast, deletion of the phosphatase regulatory subunits Cdc55 and Tpd3, or overexpression of Sit4, a PP2A-related phosphatase, confers partial rapamycin-resistance (Di Como and Arndt 1996; Jiang and Broach 1999). Mutation in a phosphatase-binding protein, Tap42, or deletion of a Tap42-binding partner, Tip41, also confer resistance to rapamycin (Di Como and Arndt 1996; Jacinto et al. 2001; Jiang and Broach 1999). While further study is required to fully understand the role of phosphatases and phosphatase-associated proteins in TOR signaling, the working hypothesis is that TOR regulates the association of Tap42 with PP2A-type phosphatases, either via modulation of the phosphorylation of Tap42 itself (only the phosphorylated form is able to interact with the phosphatase), or that of Tip41 (Jacinto et al. 2001). In both models, the Tap42-phosphatase complex is presumed to be inactive, or to exhibit differing substrate specificity. Rapamycin treatment disrupts the Tap42-phosphatase complex, leading to the association of the phosphatase catalytic subunit with other regulato-

ry subunits, and a consequent alteration in substrate specificity or activation (e.g., Jacinto et al. 2001).

In recent years, it has become clear that the activity of the TOR pathway is controlled to a large degree by nutrient availability, both in yeast and mammals (Schmelzle and Hall 2000). In yeast, treatment with rapamycin elicits the same cellular responses as nutrient deprivation, and affects the same subsets of genes controlled by nitrogen availability (Beck and Hall 1999; Cardenas et al. 1999; Hardwick et al. 1999). In mammals, the effects of rapamycin on the translation apparatus (see below) are mimicked by removal of amino acids (and particularly leucine) from the growth medium. This important topic is reviewed in detail in the accompanying chapter by Proud (see the chapter by Proud, this volume), and in recent reviews elsewhere (e.g., Kimball and Jefferson 2000). In addition to serving as a “sensor” for nutrients, TOR also appears to serve as an ATP sensor, and could be activated by mitogenic stimuli, either through conventional signaling cascades, or through direct activation by phosphatidic acid binding (reviewed in the chapter by Thomas, this volume). Thus, TOR integrates many different inputs to regulate translation and cell growth.

## 4.2

### Effects of Rapamycin on Translation Rates in Yeast

Rapamycin treatment leads to G1 arrest in yeast and mammalian cells (Abraham and Wiederrecht 1996). The mechanism leading to cell cycle arrest is not fully understood; however, one of the early events following rapamycin treatment is a reduction in translation rates. Since an increase in translation is necessary both for entry into and progression through the cell cycle (Brooks 1977) this decrease in translation could explain the rapamycin-induced cell cycle arrest. Treatment of *S. cerevisiae* with rapamycin induces a starvation-like response, resulting in a rapid decline in translation rates (>90%), in addition to nearly complete polysome dissociation (Barbet et al. 1996; Di Como and Arndt 1996). In particular, translation of the cyclin 3 (Cln3) mRNA is extremely sensitive to both rapamycin treatment and nutritional deprivation (Barbet et al. 1996; Gallego et al. 1997). Overexpression of a recombinant Cln3 mRNA (with the coding region of Cln3 fused to the 5'UTR of the rapamycin-insensitive UBI4 mRNA) rescues the rapamycin-induced cell cycle arrest (Barbet et al. 1996). How the translation of Cln3 (and of other mRNAs)

is modulated by rapamycin is unclear, however. Danaie et al. demonstrated that the translation of Cln3 is hypersensitive to inactivating mutations of eIF4E (which also causes a G1 arrest), and that a fusion with the UBI4 5'UTR confers resistance to mutations in the eIF4E gene (Danaie et al. 1999). Whether yeast eIF4E is a target of rapamycin remains to be determined, but its binding partner, the initiation factor eIF4G, is rapidly degraded following rapamycin treatment or amino acid deprivation (Berset et al. 1998; Kuruvilla et al. 2001; Powers and Walter 1999). In addition, deletion of an *S. cerevisiae* eIF4E-binding protein, Eap1p (which is not conserved in higher eukaryotes), confers partial resistance to rapamycin (Cosentino et al. 2000). The effects of rapamycin on the function of the translation apparatus itself are described below.

### 4.3

#### Effects of Rapamycin on Translation Rates in Mammals

As opposed to the dramatic effect of rapamycin on translation rates in yeast, rapamycin has a rather modest effect on global translation rates in most mammalian cells. The magnitude of this effect varies greatly amongst the various cell types (and cell lines) studied, and rapamycin is much more potent at preventing an increase in translation rates stimulated by nutrients, growth factors, cytokines, or hormones, than at reducing basal translation rates. Rapamycin has virtually no effect on IRES-mediated, cap-independent, translation, but reduces cap-dependent translation by 15%–30% in NIH 3T3 cells (Beretta et al. 1996). Recently, Grolleau et al. applied a combination of polysome profiling and microarray analysis to E6-1 Jurkat cells (a human T cell lymphoma line) treated with rapamycin. In E6-1 Jurkat cells, overall translation rates are decreased by ~35% and ~45% after 4 h and 8 h of treatment, respectively (Grolleau et al. 2002). Consistent with these observations, the translation of most mRNAs is partially decreased following rapamycin treatment. However, translation of about 6% of mRNAs (of the more than 2000 mRNAs screened) was inhibited by over 90% by rapamycin (Grolleau et al. 2002). These mRNAs include several 5'TOP-containing mRNAs (ribosomal subunits and elongation factors), as well as mRNAs lacking a 5'TOP, including those coding for several RNA-binding proteins, metabolic enzymes, and proteasome subunits (Grolleau et al. 2002).

Amongst mRNAs translated in a cap-dependent manner, there is a wide range in the degree of inhibition elicited by rapamycin treatment. Many of the same mRNAs that are hypersensitive to growth factor or hormone treatment are also extremely sensitive to rapamycin. An oft-cited example is the effect of rapamycin on 5'TOP containing mRNAs (described above and in Fumagalli and Thomas 2000; Meyuhas and Hornstein 2000). (However, the magnitude and significance of the effects of rapamycin on TOP translation have been questioned; see Hornstein et al. 2001; Tang et al. 2001). Several types of non-5'TOP-containing mRNAs are also translationally regulated by rapamycin. The translation of the IGF-II leader 3 mRNA is sensitive to rapamycin due to a specific 5'UTR sequence; a family of inhibitory RNA binding proteins can bind to the IGF-II leader 3 (but not to IGF-II leader 4, which differs only in the 5'UTR, and which is expressed constitutively), and are good candidates to mediate this inhibition (Nielsen et al. 1995, 1999). There are likely many more undiscovered examples of this type of regulation by rapamycin. A third category of rapamycin-sensitive mRNAs are those presumed to require high amounts of eIF4F helicase activity, due to the presence of long, structured 5'UTRs (as described above). Indeed, the translation of two such mRNAs, those coding for ODC and c-myc, is strongly inhibited by rapamycin treatment (Pyronnet et al. 2000; West et al. 1998).

## 5

### Translation Components Regulated via the TOR Pathway

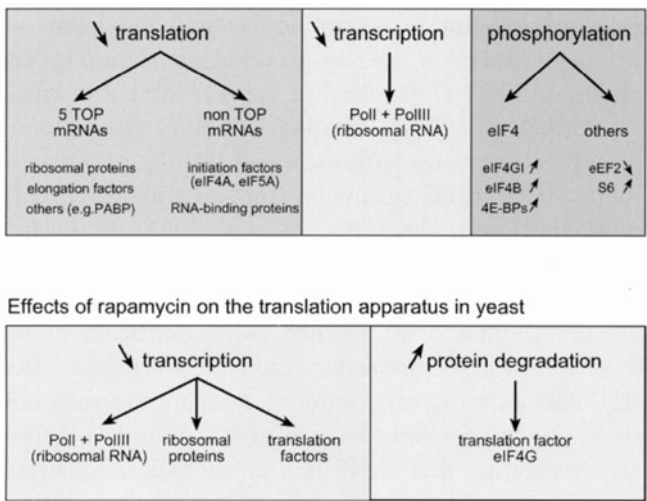
#### 5.1

##### Control of the Abundance of the Translation Machinery by the TOR Pathway

As mentioned above, rapamycin treatment inhibits the synthesis of the translation apparatus in mammals, due in part to the translational repression of the 5'TOP containing mRNAs. However, unlike the mammalian ribosomal protein mRNAs, yeast ribosomal protein mRNAs lack a 5'TOP. In spite of this, rapamycin also modulates the abundance of the translation machinery in yeast, via a number of other mechanisms. In *S. cerevisiae*, rapamycin rapidly inhibits the transcription of ribosomal protein mRNAs; the mRNA levels of all ribosomal proteins tested decrease by more than 50% after 15 min of exposure to the drug (Powers

and Walter 1999). This finding was also confirmed in several cDNA array experiments, in which the transcription of mRNAs encoding for ribosomal proteins was strongly repressed by rapamycin treatment (e.g., Cardenas et al. 1999; Hardwick et al. 1999). The mRNA levels of several other translation machinery components tested (eEF1A, eIF4E, PAPB, eIF4G, and PRT1) are also reduced to some extent in response to rapamycin treatment, with slower kinetics (Powers and Walter 1999). Importantly, rapamycin also prevents the induction in ribosomal protein mRNA transcription following a shift from a poor to high quality nutrient source (Powers and Walter 1999). Rapamycin additionally controls ribosome biosynthesis via the inhibition of Pol I and Pol III, which are responsible for the synthesis of ribosomal RNA (Powers and Walter 1999; Zaragoza et al. 1998), and the processing of the 35S precursor ribosomal RNA (Powers and Walter 1999), respectively. In addition to regulating the transcription of the mRNAs encoding translation machinery components, the TOR pathway has been implicated in regulating the proteolysis of at least one yeast translation initiation factor, eIF4G (Berset et al. 1998; Kuruvilla et al. 2001; Powers and Walter 1999). The kinetics of eIF4G degradation following rapamycin treatment coincide with the translation inhibition, as detected by [ $^{35}$ S]methionine treatment, suggesting a link between these events (Berset et al. 1998; Powers and Walter 1999). While the precise mechanism by which the TOR pathway controls the transcription and stability of translation components is not known, at least part of this effect appears to be mediated by GATA-type transcription factors (Nil1p, Gln3p; Kuruvilla et al. 2001). A summary of the known effects of rapamycin on the biosynthesis of the translation apparatus in yeast is depicted in Fig. 2.

The regulation of Pol I and Pol III transcription by rapamycin appears to be conserved in mammalian cells, where it may provide an additional level of control for the production of the components of the protein synthetic machinery (Leicht et al. 1996; Mahajan 1994). A decrease in the transcription of ribosomal protein mRNAs following rapamycin treatment has not been reported to date, but the transcription of at least one translation factor, eIF4E, has been shown to decrease following rapamycin treatment (Grolleau et al. 2002). In addition to the 5'TOP containing mRNAs, whose translation decreases following rapamycin treatment in Jurkat cells, the translation of other RNA binding proteins, including the translation initiation factors eIF4AI and eIF5A, are also



**Fig. 2** Summary of the reported effects of rapamycin on the biosynthesis and activity of the translation machinery

strongly reduced following rapamycin treatment (Grolleau et al. 2002; summarized in Fig. 2).

## 5.2 Control of the Activity of the Translation Machinery by the TOR Pathway

In higher eukaryotes, in addition to regulating the abundance of the protein synthetic machinery itself, the TOR signaling pathway directly modulates the activity of several translation factors, and other proteins involved in translation. This control has been the topic of several recent reviews, and will thus only be summarized here briefly (see also Fig. 2).

The first translation-related protein whose phosphorylation was demonstrated to be regulated by mTOR is the ribosomal S6 protein, a component of the small ribosomal subunit. S6 is phosphorylated in vivo by the kinases S6K1 and S6K2, two kinases whose activity is sensitive to rapamycin, and regulated by mTOR (Fumagalli and Thomas 2000). The phosphorylation of S6 positively correlates with the growth status of the cell, although it is unclear how phosphorylation alters S6 activity.

In recent years, it also became abundantly clear that the mTOR pathway regulates the phosphorylation of several additional components of the translation machinery. Several of the eIF4 group of translation initiation factors (see above; and Fig. 1), involved in the recruitment of ribosomes to mRNA, are targets of mTOR. Phosphorylation of two of these factors, eIF4GI and eIF4B, correlates with increased translation rates in response to hormone or mitogen treatment, and is sensitive to rapamycin treatment (Peiretti et al. 2002; Raught et al. 2002). While the identity of the kinase responsible for eIF4GI phosphorylation is unknown, the kinase that phosphorylates eIF4B appears to be S6K (reviewed in Fumagalli and Thomas 2000). In vitro, S6K1 specifically phosphorylates eIF4B on a serum-responsive, rapamycin-sensitive site (Peiretti et al. 2002). Furthermore, expression of a rapamycin-resistant form of S6K1 confers rapamycin-resistance to eIF4B phosphorylation (Peiretti et al. 2002), indicating that eIF4B is a physiological substrate for S6K. Three rapamycin-sensitive sites have been identified on eIF4GI (Raught et al. 2002), but their function in modulating the activity of the protein is unknown.

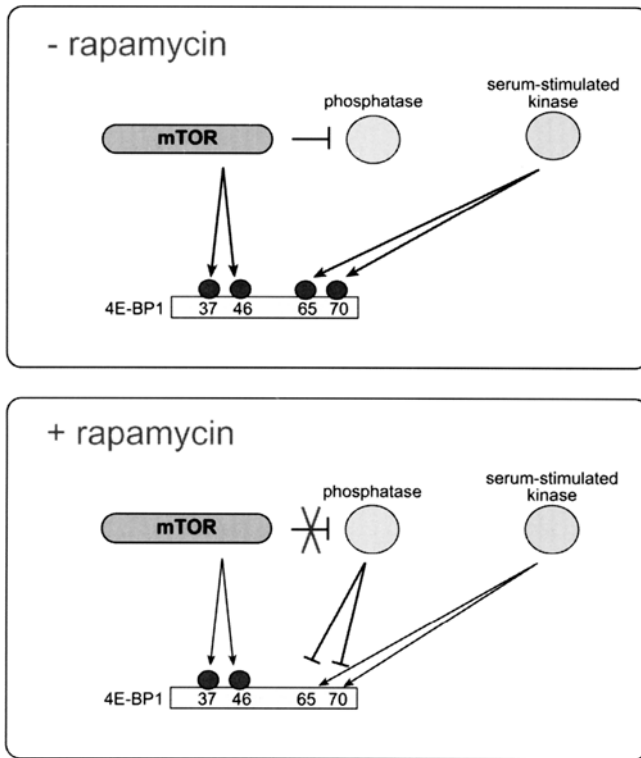
eIF4F complex formation is inhibited by rapamycin treatment (Beretta et al. 1996; Graves et al. 1995). Members of a family of translation inhibitors, the eIF4E-binding proteins (4E-BPs), bind to and sequester eIF4E from the translation machinery in a reversible manner (Gingras et al. 1999; Lin et al. 1994; Pause et al. 1994; Raught et al. 2000). While hypophosphorylated 4E-BPs possess a strong affinity for eIF4E, hyperphosphorylated 4E-BPs do not. Thus, 4E-BP hyperphosphorylation (elicited following exposure of cells to various stimuli, such as nutrients, mitogens, growth factors, or hormones) results in the dissociation of the 4E-BPs from eIF4E. Hyperphosphorylation of the 4E-BPs following stimulation, and the subsequent release of eIF4E, is prevented by rapamycin treatment. The signaling pathway leading to 4E-BP1 phosphorylation and release from eIF4E following hormone or growth factor stimulation involves activation of PI3K, which, in turn, activates the proto-oncogene serine/threonine kinase Akt (Gingras et al. 2001; Kohn et al. 1998). The tumor suppressor and phosphatase PTEN (phosphatase and *tensin* homolog, deleted from chromosome 10), which dephosphorylates the lipid products of PI3K, negatively regulates this pathway: deletion of PTEN results in increased phosphorylation of 4E-BP1 (Wu et al. 1998).

The phosphorylation of 4E-BP1 occurs on at least six residues (Thr37, Thr46, Ser65, Thr70, Ser83, and Ser112; numbering according to the hu-



man sequence; 26 l, Heesom et al. 1998). In HEK 293 cells, two primary subsets of phosphorylation sites are detected: Thr37 and Thr46 are phosphorylated in serum-starved cells, and their phosphorylation is only mildly affected upon serum-stimulation, Akt activation, or treatment with rapamycin or PI3K inhibitors (Gingras et al. 1999, 2001; von Manteuffel et al. 1996). In contrast, the phosphorylation of Ser65 and Thr70 is barely detectable in serum-deprived cells, but is greatly increased in a rapamycin-sensitive manner following serum-stimulation (Gingras et al. 2001). The phosphorylation of Thr37 and Thr46 (and possibly Ser65 and Thr70) can be achieved *in vitro* using mTOR immunoprecipitates (Brunn et al. 1997a, b; Burnett et al. 1998; Gingras et al. 1999; Mothe-Satney et al. 2000). It appears counter-intuitive that Thr37 and Thr46 are the least sensitive to rapamycin treatment. However, the kinase activity of mTOR itself does not appear to be particularly sensitive to rapamycin, either (for a discussion, see Gingras et al. 2001; Peterson et al. 1999). The function of the phosphorylation of Thr37 and Thr46 is likely to serve as “priming” sites to permit the subsequent phosphorylation of Thr70 and Ser65, as mutation of either residue abrogates phosphorylation of both Ser65 and Thr70. Phosphorylation of 4E-BP1 in serum-stimulated 293 cells proceeds in an ordered manner: phosphorylation first occurs on Thr37 and Thr46, followed by phosphorylation at Thr70, and finally, at Ser65. Since phosphorylation at Ser65 alone is not sufficient to disrupt the interaction with eIF4E (however, see evidence to the contrary in Karim et al. 2001), phosphorylation of Thr37, Thr46, Thr70, and possibly other sites are thought to synergize to effect release (discussed in Gingras et al. 2001).

The effects of rapamycin on the phosphorylation of the two most rapamycin-sensitive sites, Ser65 and Thr70, are mediated via phosphatase derepression. Using a  $^{32}\text{P}$  pulse-chase methodology, we have demonstrated that  $^{32}\text{P}$  incorporated into 4E-BP1 is lost at a faster rate in rapamycin-treated cells than in untreated cells (Raught et al. 2003). Importantly, phosphopeptide mapping indicates that rapamycin treatment does not significantly alter the phosphate turnover on Thr37 and Thr46, but results in a sharp decline of  $^{32}\text{P}$  on phosphopeptides containing Ser65 and Thr70 (Raught et al. 2003). In addition, treatment of cells with the PP1/PP2A phosphatase inhibitor calyculin A prevents the dephosphorylation of Ser65 and Thr70 following rapamycin treatment (Peterson et al. 2000; Raught et al. 2003). Thus, mTOR regulates the phosphorylation of 4E-BP1 via at least two mechanisms: phosphorylation of



**Fig. 3** mTOR differentially regulates the phosphorylation of 4E-BP1 at Thr37/Thr46 and Ser65/Thr70. A protein kinase activity present in mTOR immunoprecipitates phosphorylates 4E-BP1 at Thr37 and Thr46. This kinase activity is only mildly affected by rapamycin treatment. mTOR also regulates the phosphorylation of Ser65 and Thr70 through repression of a phosphatase directed against these sites. In the presence of rapamycin, the phosphatase is derepressed and dephosphorylates Ser65 and Thr70

Thr37 and Thr46, and prevention of the dephosphorylation of Ser65 and Thr70 via phosphatase repression (summarized in Fig. 3).

Taken together, these data indicate that the proteins mediating the binding of ribosomes to mRNA are key targets for mTOR regulation. While there is no data linking the activity of other initiation factors to the mTOR pathway, at least one elongation factor is phosphorylated in a rapamycin-dependent manner. Insulin signaling, in addition to eliciting an increase in translation initiation, induces an increase in elongation

rates. In CHO cells, this is accompanied by dephosphorylation of the translation elongation factor eEF2 (Redpath et al. 1996). Phosphorylation of eEF2 near its N-terminus prevents its activity, likely by inhibiting the interaction of eEF2 with the ribosome (Proud 2000). eEF2 dephosphorylation following insulin treatment is prevented by rapamycin, indicating a role for mTOR in controlling both the initiation and elongation phases of protein synthesis (Redpath et al. 1996).

In summary, a rapamycin-sensitive signaling pathway modulates the phosphorylation of several proteins that play key roles in translation: S6, eIF4B, eIF4GI, the 4E-BPs, and eEF2. It is likely that more targets for mTOR-mediated phosphorylation will be discovered when systematic approaches are undertaken.

### 5.3

#### Negative Feedback Loop from Translation to the TOR Pathway

Inasmuch as the mTOR pathway controls the activity of the translational machinery, several pieces of evidence indicate that the translational machinery also sends signals, as a negative feedback loop, to regulate the mTOR pathway itself. For example, treatment of cells with translational inhibitors (anisomycin or cycloheximide) decreases translation rates, yet they induce hyperphosphorylation of both S6K1 and 4E-BP1 (e.g., Brown and Schreiber 1996; von Manteuffel et al. 1997). Whether the TOR pathway senses damage to specific components of the translational machinery caused by the translational inhibitors, or a change in translation rates, is unknown. In another example, overexpression of the initiation factor eIF4E, both in stable transfectants and in a tetracycline-inducible cell line, results in a sharp dephosphorylation of S6K1 and 4E-BP1 (Khaleghpour et al. 1999). The extent of dephosphorylation observed is proportional to the levels of eIF4E overexpressed, and to the induction of translation of a structured mRNA (ODC; Khaleghpour et al. 1999). Interestingly, 4E-BP1 was also found to be drastically dephosphorylated in many mouse mammary tumors, as compared to their normal counterparts, indicating that negative feedback loop to mTOR is not limited to cases of eIF4E overexpression (eIF4E was not significantly overexpressed in these tumors; Raught et al. 1996). In eIF4E-transformed cells, or in the mouse mammary tumors, the negative feedback loop onto the mTOR pathway is clearly not sufficient to block the increase in translation rates, or the transformation phenotype (Khaleghpour et al.

1999; Lazaris-Karatzas et al. 1990; Raught et al. 1996). However, such a feedback mechanism could be crucial to control the activity of the translational machinery in more physiological conditions.

## 6

### Perspectives

A better understanding of the mTOR signaling pathway would be beneficial to human health. Rapamycin is currently used in the clinic as an immunosuppressant, as it is effective at preventing rejection in kidney transplant patients when used in combination with cyclosporin A (CsA; Kahan 2000). Due to the synergistic effects of rapamycin and CsA on the human immune system, the use of rapamycin in combination therapy has allowed for a significant reduction in CsA doses. This, in turn, has led to reduced CsA-associated nephropathy (Kahan 2001). However, rapamycin itself is not without adverse effects, the most widely recognized being hyperlipidemia (occurring in about 40% of the renal transplant recipients), which can exacerbate the lipid and sterol disorders caused by CsA, as well as the renal disease (Kahan 2001). A deeper knowledge of the molecular mechanism of action of mTOR should allow for a better understanding of the synergy between CsA and rapamycin, and for the development of drugs directed against downstream targets of mTOR (possibly translational targets).

There is a considerable body of data linking the abundance of initiation factors such as eIF4E, eIF4GI, eIF4A, and eIF3 with cancer. Elevated levels of several initiation factors have been reported in naturally-occurring tumors (reviewed in Hershey and Merrick 2000). Furthermore, forced overexpression of eIF4E or eIF4G is sufficient to transform immortalized murine cells in culture (reviewed in Hershey and Merrick 2000). The reasons for the transformation by eIF4E may be twofold: eIF4E shortens the G1 phase of the cell cycle, thus increasing proliferation, and it acts as an antiapoptotic factor (Polunovsky et al. 2000). Conversely, overexpression of 4E-BP1 reverts the malignant phenotype of cells transformed by eIF4E, Src, or Ras (Rousseau et al. 1996), and has been found to sensitize Ras-transformed cells to apoptosis (Polunovsky et al. 1996).

In addition, components of the signaling pathway leading to the phosphorylation of the 4E-BPs, eIF4GI, eIF4B, and other translational proteins are often altered in neoplasms. For example, amplification of the

catalytic subunit of PI3K  $\alpha$  is detected in ~40% of ovarian cancers (Shayesteh et al. 1999), and Akt genes are amplified or overexpressed in several types of cancers (e.g., breast, gastric, ovarian, pancreatic, and prostate; Bellacosa et al. 1995; Cheng et al. 1992, 1996; Nakatani et al. 1999; Rousseau et al. 1996). Most strikingly, the tumor suppressor PTEN is one of the most frequent targets for mutation in human cancers. The activity of PTEN can be compromised by multiple mechanisms: somatic deletion, homozygous mutation, loss of heterozygosity with silencing of the remaining allele (by methylation-dependent and methylation-independent mechanisms), as well as posttranslational regulation of PTEN stability and activity (reviewed in Cantley and Neel 1999; Mills et al. 2001). It is likely that some of the effects of altering the PI3K pathway are mediated through changes in the activity of key translation components.

Rapamycin analogs, such as CCI-779 and RAD-001, are currently being tested as antineoplastic agents. Rapamycin and rapamycin analogs inhibit the growth of a variety of tumor cell lines in culture, and xenografts in mice. Importantly, tumors harboring mutations in PTEN are hypersensitive to rapamycin (Neshat et al. 2001; Podsypanina et al. 2001). CCI-779 inhibits the growth of PTEN-deficient cells at much lower concentrations than are required to inhibit the parental cells, indicating that rapamycin analogs could be used to selectively kill cancer cells (Neshat et al. 2001; Podsypanina et al. 2001). Furthermore, mutation or amplification of PI3K or Akt sensitizes transformed cells to rapamycin (Mills et al. 2001; Nakatani et al. 1999; Neshat et al. 2001), raising the possibility of screening for alterations in the PI3K pathway prior to determining a treatment course.

*Acknowledgements.* Work in the authors' laboratory was supported by the National Cancer Institute of Canada (NCI), the Canadian Institutes of Health Research (CIHR), and the Howard Hughes Medical Institute (HHMI) International Scholars program. A.-C.G. and B.R. were supported by postdoctoral fellowships from the CIHR. NS is a HHMI International Scholar and a CIHR Distinguished Scientist.

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# Modulation of the Protein Kinase Activity of mTOR

J. C. Lawrence<sup>1</sup> · T.-A. Lin<sup>2</sup> · L. P. McMahon<sup>1</sup> · K. M. Choi<sup>1</sup>

<sup>1</sup> Department of Pharmacology, University of Virginia School of Medicine,  
1300 Jefferson Park Avenue, Charlottesville, VA 22908-0735, USA  
*E-mail: JCL3p@virginia.edu*

<sup>2</sup> Bristol-Myers Squibb Pharmaceutical Research Institute,  
Rt. 206 and Province Line Road, Princeton, NJ 08543-4000, USA

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**Abstract** mTOR is a founding member of a family of protein kinases having catalytic domains homologous to those in phosphatidylinositol 3-OH kinase. mTOR participates in the control by insulin of the phosphorylation of lipin, which is required for adipocyte differentiation, and the two translational regulators, p70<sup>S6K</sup> and PHAS-I. The phosphorylation of mTOR, itself, is stimulated by insulin in Ser2448, a site that is also phosphorylated by protein kinase B (PKB) in vitro and in response

to activation of PKB activity in vivo. Ser2448 is located in a short stretch of amino acids not found in the two TOR proteins in yeast. A mutant mTOR lacking this stretch exhibited increased activity, and binding of the antibody, mTab-1, to this region markedly increased mTOR activity. In contrast, rapamycin-FKBP12 inhibited mTOR activity towards both PHAS-I and  $p70^{S6K}$ , although this complex inhibited the phosphorylation of some sites more than that of others. Mutating Ser2035 to Ile in the FKBP12-rapamycin binding domain rendered mTOR resistant to inhibition by rapamycin. Unexpectedly, this mutation markedly decreased the ability of mTOR to phosphorylate certain sites in both PHAS-I and  $p70^{S6K}$ . The results support the hypotheses that rapamycin disrupts substrate recognition instead of directly inhibiting phosphotransferase activity and that mTOR activity in cells is controlled by the phosphorylation of an inhibitory regulatory domain containing the mTab-1 epitope.

**Abbreviations** *cAMP* Adenosine 3',5' cyclic phosphate · *eIF* Eukaryotic initiation factor · *4E-BP1* eIF4E-binding protein 1 · *FKBP-12* FK506 binding protein of  $M_r=12,000$  · *GST* Glutathione S transferase · *HEK* Human embryonic kidney · *PHAS* Phosphorylated heat- and acid-stable eIF4E-binding protein · *mTab* mTOR antibody · *mTOR* Mammalian target of rapamycin · *PI 3-kinase* Phosphatidylinositol 3-OH kinase · *PKB* Protein kinase B · *p70<sup>S6K</sup>*  $M_r=70,000$  ribosomal protein S6 kinase

## 1

### Introduction

TOR proteins function in a nutrient-sensing pathway that maintains a balance between amino acid availability, protein synthesis, and cell growth (Schmelzle and Hall 2000). There is increasing evidence that insulin and certain growth factors control mTOR. This chapter reviews studies from our laboratory on the control of mTOR by insulin.

## 2

### mTOR-Dependent Control of PHAS-I Phosphorylation

PHAS-I (also known as 4E-BP1) is a translational repressor originally purified from rat adipocytes (Hu et al. 1994). Nonphosphorylated PHAS-I binds with high affinity to eIF4E (Lin et al. 1994; Pause et al. 1994), the mRNA cap-binding protein, and prevents the association be-

tween eIF4E and eIF4G (Gingras et al. 1999b). When phosphorylated in response to insulin, PHAS-I dissociates from eIF4E (Lin et al. 1994; Pause et al. 1994), which is then able to bind eIF4G. This interaction is necessary for formation of the eIF4F complex, which is required for efficient cap-dependent translation initiation (Gingras et al. 1999b). Our finding that rapamycin inhibited the effects of insulin on increasing PHAS-I phosphorylation and on promoting the dissociation of the PHAS-I/eIF4E complex implicated mTOR in the control of cap-dependent translation (Graves et al. 1995; Lin et al. 1995).

## 2.1

### Targets of mTOR in Adipocytes

Compared to other cells, fat cells contain very high concentrations of PHAS-I (Hu et al. 1994; Lin and Lawrence 1996). Why fat cells should have so much of this regulator of mRNA translation is not immediately obvious, but it is consistent with the role of mTOR in controlling cell growth. Although over 90% of a typical fat cell is triglyceride, these cells like any other must synthesize protein to grow, and adipocyte growth may be truly dramatic. While the net increase in protein is less than the increase in cell volume (sometimes more than 20-fold), there is cytosolic expansion, which requires protein synthesis, as does generation of membrane to surround the central lipid droplet.

The insulin-stimulated phosphorylation of lipin in adipocytes is also inhibited by rapamycin (Huffman et al. 2002). Lipin is the product of the gene that is mutated in fatty liver dystrophy (*fld*) mice (Peterfy et al. 2001). These animals exhibit several striking phenotypic abnormalities, including hyperlipidemia, fatty liver, and defects in adipocyte differentiation. The function of lipin is still not known, but it is clear from the *fld/fld* phenotype that it has an important role in fat cell biology. Rapamycin prevents fat cell differentiation in vitro (Yeh et al. 1995). Perhaps this effect is due to inhibition of mTOR signaling to lipin. While additional studies are needed to test this hypothesis and to elucidate the function of lipin, the coordination of protein and lipid synthesis in the fat cell represents a challenge that appears to be at least partially met by mTOR.



## 2.2

### Phosphorylation Sites in PHAS-I

By peptide mapping and amino acid sequencing, the following five phosphorylation sites were identified in PHAS-I in rat adipocytes: Thr36, Thr45, Ser64, Thr69, and Ser82 (Fadden et al. 1997). These assignments have been confirmed by mass spectrometric analyses (Gygi et al. 1999), by mutational analyses (Mothe-Satney et al. 2000), and by using phosphospecific antibodies (Mothe-Satney et al. 2000). The five sites conform to a Ser/ThrPro (S/TP) motif, and except for Ser82, the sites are conserved in PHAS proteins from other species.

Insulin stimulates the phosphorylation of Thr36, Thr45, Ser64, and Thr69 in adipocytes (Fadden et al. 1997; Mothe-Satney et al. 2000; Scott and Lawrence 1998) and HEK293 cells (Mothe-Satney et al. 2000a, b), even in cells incubated in buffer lacking amino acids, and rapamycin attenuates phosphorylation of these sites. Adding amino acids stimulates the phosphorylation of the same four sites in a rapamycin-sensitive manner (Mothe-Satney et al. 2000a, b). These findings support the conclusion that insulin and amino acids control PHAS-I phosphorylation by two pathways that converge on mTOR. It is not surprising that multiple pathways exist for controlling mTOR function, as both hormonal and substrate signals are often utilized in the control of important metabolic processes.

## 2.3

### Ordered Phosphorylation of PHAS-I

Mutating any one of the three Thr sites to Ala blocks phosphorylation of Ser64, indicating that the phosphorylation of PHAS-I is ordered (Gingras et al. 1999a; Mothe-Satney et al. 2000a, b). Under basal conditions, Thr36 and Thr45 are more highly phosphorylated than Thr69, but the phosphorylation of Thr36 and Thr45 is not strictly constitutive, as it is stimulated by insulin (Mothe-Satney et al. 2000a, b). Thr69 phosphorylation does not absolutely depend on the phosphorylation of other sites, since a PHAS-I protein containing Thr69, but having Ala substitutions in the other four phosphorylation sites, was still phosphorylated in an insulin- and rapamycin-sensitive manner (Mothe-Satney et al. 2000b). Moreover, removing the first 16 amino acids in PHAS-I essentially abolished phosphorylation of Thr36 and Thr45, but only modestly decreased

phosphorylation of Thr69 (Tee and Proud 2002). Curiously, while the phosphorylation of Ser64 markedly decreases the affinity of PHAS-I for eIF4E in vitro (Lin et al. 1994; Mothe-Satney et al. 2000b), mutating Ser64 to Ala had little, if any, effect on the association of PHAS-I with eIF4E (Mothe-Satney et al. 2000b). Thus, the role of Ser64 phosphorylation, as well as the mechanism and importance of the ordered phosphorylation of this site, are unclear.

### 3

#### **mTOR as a Protein Kinase**

The initial results with rapamycin implied that mTOR signaled upstream of PHAS-I (Graves et al. 1995). At the time there was reason to suspect that mTOR might act more directly. The homology between mTOR and DNA-dependent protein kinase had been noted and mTOR was known to autophosphorylate (Hunter 1995).

#### 3.1

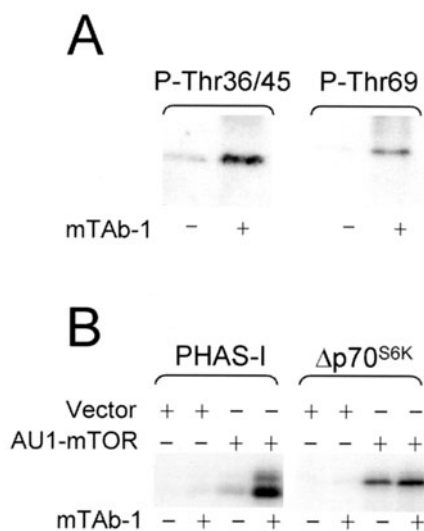
##### **Direct Phosphorylation of PHAS-I by mTOR**

mTOR immunoprecipitated from rat brain extracts with mTab-1, an antibody that recognizes a region between the kinase and FATC domains (Brunn et al. 1997a), phosphorylated recombinant PHAS-I (Brunn et al. 1997b). PHAS-I was also phosphorylated by AU1-epitope-tagged mTOR that had been overexpressed in HEK293 cells, but not by a mutant mTOR rendered kinase-dead by an Asp2338 to Ala mutation in the catalytic domain (Brunn et al. 1997b). The finding that mTOR could transfer phosphate from ATP to an exogenous substrate represented an important milestone in establishing mTOR as a bona fide Ser/Thr protein kinase.

#### 3.2

##### **mTab-1 Is an Activating Antibody**

The use of mTab-1 was fortuitous, both for detecting the protein kinase activity of mTOR and for detection of phosphorylation of mTOR, itself. The first indication that there was something unusual about this antibody was the observation that the rate of PHAS-I phosphorylation with mTab-1 was much higher than with mTOR immunopurified using



**Fig. 1A, B** Activation of mTOR by mTab1. **A** GST-mTOR (human) was overexpressed in sf9 cells by using baculovirus and affinity-purified by using glutathione-Sepharose (McMahon et al. 2002). Samples of the purified mTOR were incubated without or with mTab-1 before incubation with PHAS-I and ATP under conditions described previously (Mothe-Satney et al. 2000a). Phosphorylation was detected by using phosphospecific antibodies (Mothe-Satney et al. 2000a). **B** 293T cells were transfected with vector alone (pcDNA3) or an AU1-mTOR (rat) expression construct. After immunoprecipitations with anti-AU1 antibody, samples were incubated with and without mTab-1 before kinase assays were conducted using [ $\gamma$ - $^{32}$ P]ATP as described previously (Mothe-Satney et al. 2000a). Equal amounts (1  $\mu$ g/reaction) of PHAS-I or a truncated p70<sup>S6K</sup> protein were used as substrates (McMahon et al. 2002). Autoradiograms of the  $^{32}$ P-labeled proteins are shown

mTab-2, an antibody to a region just upstream of the FAT domain (Brunn et al. 1997a). Subsequently, we found that adding mTab-1 to mTOR that had been isolated from brain extracts with mTab-2 or affinity-purified with a FKBP12-rapamycin resin dramatically increased kinase activity (Brunn et al. 1997a). mTab-1 also increased the kinase activity of both recombinant AU1-mTOR after isolation from 293T cells (Mothe-Satney et al. 2000a) and GST-mTOR purified from sf9 cells after baculovirus expression (Fig. 1). Thus, mTab-1 is an activating antibody.

### 3.3

#### mTab-1 Binds to an Inhibitory Regulatory Domain in mTOR

The region of mTOR containing the mTab-1 epitope appears to function as an inhibitory domain, since deleting the epitope results in constitutive activation of mTOR with respect to PHAS-I phosphorylation (Sekulić et al. 2000). Presumably, mTab-1 neutralizes the influence of the inhibitory domain. We have recently investigated the influence of the mTab-1 epitope on the phosphorylation of sites in PHAS-I and in a form of p70<sup>S6K</sup> containing the COOH-terminal half of the protein (McMahon et al. 2002), which includes Thr389 but lacks the kinase domain (Burnett et al. 1998). Without mTab-1, this p70<sup>S6K</sup> protein was phosphorylated much more efficiently than PHAS-I (Fig. 1; McMahon et al. 2002). mTab-1 had little effect on the phosphorylation of the p70<sup>S6K</sup> protein. Similarly, deleting the mTab-1 epitope increased PHAS-I phosphorylation, but had a relatively small effect on the phosphorylation of p70<sup>S6K</sup> (McMahon et al. 2002). The rates of phosphorylation of PHAS-I and the p70<sup>S6K</sup> protein by mTab-1-treated mTOR were comparable (Fig. 1).

### 3.4

#### Sites Phosphorylated by mTOR

In our initial characterization we found that mTOR phosphorylated Thr36, Thr45, Ser64, and Thr69 (Brunn et al. 1997b). Subsequently, other groups failed to observe phosphorylation of the latter two sites (Burnett et al. 1998; Gingras et al. 1999a). We resolved this discrepancy in a series of experiments with recombinant epitope-tagged mTOR. With AU1-mTOR isolated from 293T cells by using anti-AU1 antibody, Thr36 and Thr45 were the major sites phosphorylated. However, incubating anti-AU1 antibody-purified mTOR with mTab-1 resulted in dramatic increases in the phosphorylation of Thr69 and Ser64 (Mothe-Satney et al. 2000a). Phosphorylation of both of these sites was abolished by rapamycin-FKBP12, but neither site was phosphorylated by an AU1-mTOR rendered kinase-dead by an Asp2338 to Ala mutation (McMahon et al. 2002; Mothe-Satney et al. 2000a). These findings confirm that mTOR is capable of phosphorylating Thr69 and Ser64, and demonstrate that the activating antibody, mTab-1, accounts for the discrepant results.

Another issue concerning the specificity of mTOR arises from differences in the nature of the sites phosphorylated. Thr229, Ser371, Thr389,

Ser404, and Ser411 in p70<sup>S6K</sup> are sensitive to rapamycin in cells (Dennis et al. 1996; Saitoh et al. 2002). Of these sites, Ser371 (Saitoh et al. 2002) and Thr389 (Burnett et al. 1998) have been found to be phosphorylated by mTOR in vitro. Ser371 and Ser411 are followed by Pro, like the five sites in PHAS-I. In contrast, Thr229, Thr389, and Ser404 are flanked by hydrophobic residues. Based on studies of the ACG family of protein kinases (Hunter 1995), it would be very unusual for a protein kinase to phosphorylate sites as different as the hydrophobic sites in p70<sup>S6K</sup> and the (S/T)P sites in PHAS-I. However, it may not be reasonable to expect mTOR to conform to the rules for ACG kinases, given mTOR's homology to PI 3 kinase, which can phosphorylate both lipid and protein (Bondeva et al. 1998).

#### 4

### Control of mTOR

Studies in several cell types indicate that mTOR phosphorylation and/or activity are controlled by serum (Burnett et al. 1998), insulin (Navé et al. 1999; Scott et al. 1998a), and certain growth factors (Sekulić et al. 2000).

#### 4.1

### Insulin-Stimulated Phosphorylation of mTOR

An unexpected result with mTAB-1 led to the discovery of insulin-stimulated phosphorylation of mTOR. Incubating cells with insulin decreased mTAB-1 binding to mTOR (Scott et al. 1998a). Immunoreactivity with mTAB-1 was restored by treating mTOR from insulin-treated cells with protein phosphatase, implying that insulin stimulated the phosphorylation of one or more sites within the mTAB-1 epitope (Scott et al. 1998a). The finding that insulin decreased mTAB-1 binding by as much as 90% is indicative of a high stoichiometry of phosphorylation.

The mTAB-1 epitope contains two sites, Thr2446 and Ser2448, which are consensus motifs for phosphorylation by PKB (Navé et al. 1999; Scott et al. 1998a; Sekulić et al. 2000). Results obtained with a conditionally active PKB, which consisted of PKB fused to a mutant estrogen receptor (Kohn et al. 1998), provided direct evidence that mTOR was phosphorylated in response to PKB activation (Kohn et al. 1998; Scott et al. 1998a). In fibroblasts overexpressing the conditionally active kinase, mTAB-1 binding to mTOR was decreased and phosphorylation of

PHAS-I were increased within minutes of treating the cells with tamoxifen (Kohn et al. 1998; Scott et al. 1998a), which leads to activation of the PKB fusion protein. More recent results with phosphospecific antibodies have confirmed that Ser2448 is phosphorylated in response to insulin and demonstrated that PKB is able to directly phosphorylate this site in vitro (Navé et al. 1999; Reynolds et al. 2002; Sekulić et al. 2000).

## 4.2

### Activation of mTOR by Insulin

With the identification of PHAS-I as a substrate, it became possible to assess directly the control of mTOR activity. mTab-2 was used to investigate the effects of insulin on mTOR activity, because mTab-1 activates mTOR and its binding to mTOR is inhibited by insulin (Brunn et al. 1997; Scott et al. 1998a). Incubating 3T3-L1 adipocytes with insulin increased the PHAS-I kinase activity of mTab-2 immunoprecipitated mTOR by three- to fourfold (Scott et al. 1998a). The insulin-stimulated activity in mTab-2 immune complexes was inhibited by rapamycin-FKBP12 and abolished by wortmannin, as would be expected of an mTOR-mediated process (Scott et al. 1998a). Treating immune complexes with purified protein phosphatase abolished the effect of insulin, indicating that the activation was due to increased phosphorylation of mTOR (Scott et al. 1998a). The increase in activity produced by insulin was similar in magnitude to the increase in PHAS-I phosphorylation produced by insulin in intact cells.

## 4.3

### Inhibition of mTOR by cAMP and by Methylxanthines

Important signaling pathways are typically controlled by both stimulatory and inhibitory inputs, and there is evidence that mTOR signaling is subject to inhibition by physiological stimuli. Increasing cAMP in 3T3-L1 adipocytes and certain other cell types inhibits the phosphorylation of PHAS-I and attenuates the activation of p70<sup>S6K</sup> (Graves et al. 1995; Monfar et al. 1995; Scott and Lawrence 1998). Increasing cAMP also attenuated the effect of insulin on increasing mTOR phosphorylation in 3T3-L1 adipocytes; however, it did not inhibit the activation of PKB produced by insulin (Scott and Lawrence 1998), implying that the phosphorylation of Ser2448 or another site affecting mTab-1 binding is mod-

ulated by a PKB-independent mechanism. cAMP phosphodiesterase inhibitors were not required to observe effects of agents that increase cAMP on the mTOR pathway. This is an important point because theophylline, caffeine, and certain other methylxanthines directly inhibit mTOR activity in vitro (McMahon et al. 2002; Scott and Lawrence 1998).

#### 4.4

##### Is Ser2448 Phosphorylation Important?

The results with the activating antibody (Brunn et al. 1997a; Mothe-Satney et al. 2000a), the stimulatory effects of deleting the antibody epitope on mTOR activity (Sekulić et al. 2000), the insulin-stimulated phosphorylation of Ser2448 in the epitope (Navé et al. 1999; Reynolds et al. 2002; Scott et al. 1998a; Sekulić et al. 2000), and the dephosphorylation of mTOR in response to cAMP (Scott and Lawrence 1998) are consistent with the hypothesis that phosphorylation of Ser2448 increases mTOR activity. However, all of this evidence is correlative. Unfortunately, the inability to phosphorylate Ser2448 efficiently in vitro has hindered direct experiments to determine whether phosphorylation activates mTOR.

Sekulić et al. (Sekulić et al. 2000) have investigated the role of Ser2448 phosphorylation in the control of p70<sup>S6K</sup>. HEK293 cells were cotransfected with FLAG-tagged p70<sup>S6K</sup> and a mutant mTOR having a Ser2035 to Ile mutation, which abolishes high affinity binding of rapamycin-FKBP12. The cells were then treated with rapamycin to block endogenous mTOR. The transfected rapamycin-resistant mTOR supported insulin-stimulated phosphorylation of p70<sup>S6K</sup>. Essentially identical activation of p70<sup>S6K</sup> was supported by a rapamycin-resistant mTOR having a Ser2448 to Ala mutation. These findings certainly provide cause to question the role of Ser2448 phosphorylation in the control of p70<sup>S6K</sup>. However, as noted above, our more recent studies indicate that mTab-1 (Fig. 1) and deletion of the mTab-1 epitope (McMahon et al. 2002) have relatively little influence on the phosphorylation of p70<sup>S6K</sup>. Thus, p70<sup>S6K</sup> might not have been the most sensitive target for evaluating the role of Ser2448 phosphorylation. In our view, the question of whether or not Ser2448 phosphorylation controls mTOR activity should remain open.

## 5

**Influence of the FRB on mTOR Activity In Vitro**

In interpreting the results of studies in which mTOR rendered rapamycin-resistant by mutation of Ser2035 is overexpressed in cells, it is generally assumed that rapamycin treatment abolishes the effect of endogenous mTOR, and that except for sensitivity to rapamycin, the mutant mTOR behaves like the wild-type endogenous mTOR. Neither assumption is safe. FKBP12-rapamycin has a relatively small inhibitory effect on the phosphorylation of Thr36 and Thr45 in PHAS-I by mTOR in vitro (Mothe-Satney et al. 2000a), indicating that rapamycin does not ablate the function of mTOR. More recently, we have found that mTOR with the Ser 2035 to Ile mutation had dramatically lower activity than wild-type mTOR with respect to the phosphorylation of Thr36 and Thr45 (McMahon et al. 2002). Interestingly, the ability of this mutant mTOR to phosphorylate Thr69 was only modestly impaired. The Ile mutation also decreased phosphorylation of Thr389 in a truncated p70<sup>S6K</sup>, and several other substitutions at position 2035 almost abolished phosphorylation of this site (McMahon et al. 2002). In contrast, a Trp mutation in this position conferred almost complete resistance to rapamycin and only slightly decreased the ability of mTOR to phosphorylate PHAS-I and p70<sup>S6K</sup>. This rapamycin-resistant form of mTOR should be better than the Ser2035 to Ile mutant for investigating the function of mTOR in cells.

## 6

**Working Hypothesis**

mTOR contains a substrate recognition domain, distinct from the active site of the kinase, that appears to be in or near the FRB, since mutations in Ser2035 have dramatic effects on the sites in PHAS-I and p70<sup>S6K</sup> that are phosphorylated (McMahon et al. 2002). Two groups have very recently described a scaffolding protein, termed raptor, that interacts with mTOR (Hara et al. 2002; Kim et al. 2002). Interestingly, raptor binds PHAS-I and appears to be required for the phosphorylation of PHAS-I, but not for the phosphorylation of p70<sup>S6K</sup> (Hara et al. 2002). Thus, the selective activation of PHAS-I phosphorylation by mTAb-1 would be consistent with an action of the regulatory domain to prevent PHAS-I binding to the mTOR-raptor complex, or to block presentation of rap-



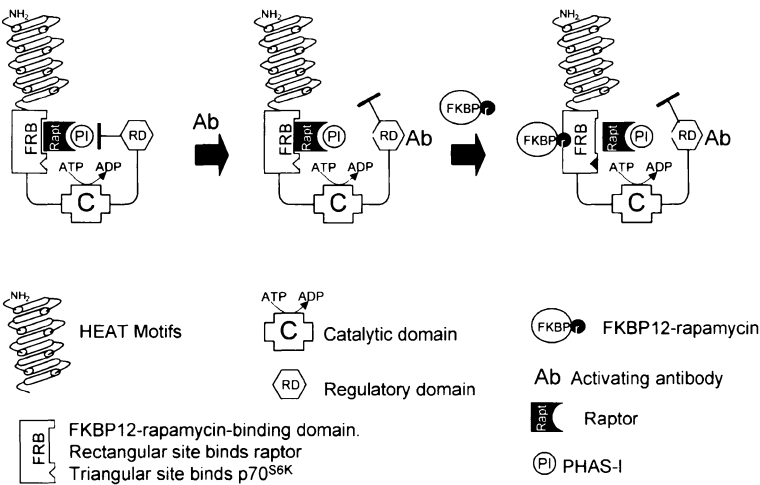


Fig. 2 Control of mTOR activity by mTab-1 and rapamycin

tor-bound PHAS-I to the catalytic site (Fig. 2). The findings of Kim et al. (2002) indicate that the effects of rapamycin are mediated, at least in part, by lessening the strength of the interaction between raptor and mTOR. However, failure of rapamycin-FKBP12 to promote complete dissociation of raptor might explain why the phosphorylation of Thr36 and Thr45 in PHAS-I are resistant to inhibition by rapamycin. In this model, rapamycin-FKBP binding would change the p70<sup>S6K</sup> binding site to prevent phosphorylation of Thr389 in p70<sup>S6K</sup>. Obviously, more work is needed to define mechanisms involved in the interactions between mTOR and its substrates.

7

### Concluding Remarks

While nutrient availability is a factor determining cell growth in all organisms, growth factors exert an additional level of control in mammals. Most studies of the regulation of mTOR in cells have relied upon measurements of changes in downstream targets of mTOR, and we still know relatively little with respect to the mechanisms involved in the control of mTOR itself. Continued investigation of the role of raptor will undoubtedly shed new light on signaling by mTOR. We believe that phosphoryla-

tion of Ser2448 will prove in some way to be important in the control of mTOR, and investigating the possible role of this phosphorylation site in control substrate presentation by raptor will be important. However, it would be naïve to believe that such a large and important protein as mTOR would be controlled by phosphorylation of a single site by a single protein kinase. Defining the mechanisms by which mTOR integrates hormonal and nutrient signals is a challenge for future investigations.

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# Role of mTOR Signalling in the Control of Translation Initiation and Elongation by Nutrients

C. G. Proud

Division of Molecular Physiology, School of Life Sciences, University of Dundee,  
MSI/WTB Complex, Dundee, DD1 5EH, UK

*E-mail: c.g.proud@dundee.ac.uk*

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**Abstract** Protein synthesis requires nutrients both as precursors (amino acids) and as a source of energy, since this process consumes a high proportion of cellular metabolic energy. Recent work has shown that both types of nutrients directly influence the activities of components of the translational machinery in mammalian cells. Amino acids positively regulate signalling through the mammalian target of the rapamycin (mTOR) pathway, although the degree of dependency on external amino

acids varies between cell types. mTOR signalling modulates several key components involved in mRNA translation, in particular (via repressor proteins) the cap-binding initiation factor eIF4E, the ribosomal protein S6 kinases, and elongation factor eEF2. The branched-chain amino acid leucine is the most effective one in most cell types. It is currently unclear how mammalian cells sense prevailing amino acid levels, although this may involve intracellular amino acids. Cellular ATP levels can also influence mTOR activity. The activities of some translation factors are modulated by mTOR-independent mechanisms. Examples include the regulation of eEF2 by cellular energy levels, which may be controlled via the AMP-activated protein kinase, and the activity of the guanine nucleotide-exchange factor eIF2B, which is modulated by amino acids and metabolic fuels.

## 1

### **Introduction**

It has long been known that starvation or lack of nutrients influence protein synthesis rates in cells and tissues. This is not unexpected given that protein synthesis requires both amino acids, as precursors, and metabolic energy. Protein synthesis is a major energy consuming process. Studying the molecular mechanisms involved in the regulation of protein synthesis required a better understanding of the mechanism of translation and its control. Discoveries over the last 10 years or so have facilitated studies at the molecular level into the regulation of protein synthesis by nutrients, and the interplay between nutrients and hormonal signals. An important discovery in the last few years is that a number of components of the translational machinery in mammalian cells are subject to acute regulation by the nutrient status of the cell. Regulation of most of these components is linked to the rapamycin-sensitive mTOR signalling pathway. These targets for mTOR signalling include regulators of translation initiation and elongation, and protein kinases acting on the small ribosomal subunit. This knowledge has allowed investigators to return to the key issue of placing this improved knowledge in a physiological context, and studying the regulation of protein synthesis by nutrients in physiologically important tissues such as skeletal muscle. This chapter reviews our current understanding of the regulation of translation factors by nutrients, via the mTOR pathway, and recent studies applying this information to tissues such as skeletal muscle and heart.

## 2

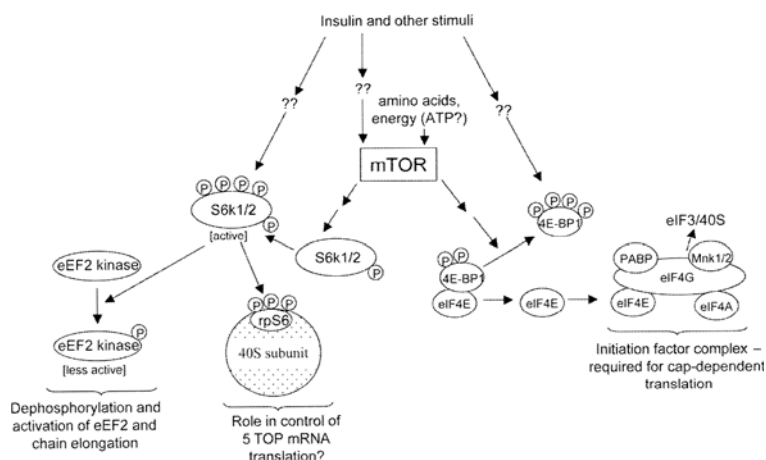
**Regulation of Translation Initiation Factor Complex Formation**

Many studies have focused on skeletal muscle, as it is a tissue of particular importance for whole body protein metabolism. In early work, overnight fasting led to disaggregation of polyribosomes in rat skeletal muscle (Morgan et al. 1971). This indicates an impairment of the stage of protein synthesis in which ribosomes bind to the mRNA, i.e. translation initiation. Longer term fasting involves an additional effect, a reduction in the levels of ribosomes in the tissue, manifested as a fall in its RNA content (the bulk of cellular RNA is ribosomal RNA; Li et al. 1979).

## 2.1

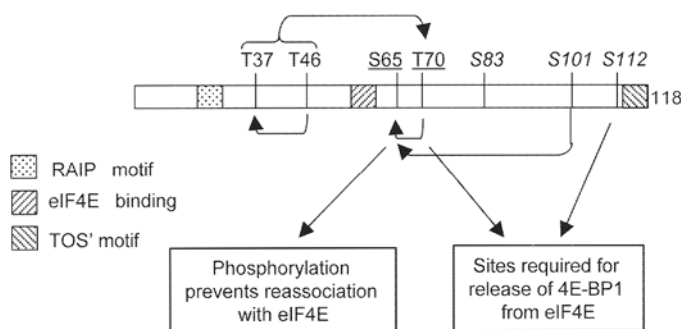
**Regulation of eIF4E by eIF4E-Binding Proteins**

The eukaryotic initiation factor (eIF) 4E binds to the 5'-cap structure of eukaryotic mRNAs and may provide the first contact between the translational machinery and the mRNA in *de novo* translation initiation. eIF4E also interacts with several types of protein binding partners. One class comprises the scaffold proteins of the eIF4G group, of which two exist in yeast and two are known in mammals. eIF4G interacts with a number of other proteins. These include the helicases (in mammals, eIF4A<sub>I</sub> and eIF4A<sub>II</sub>); the poly(A)-binding protein, PABP; the multisubunit initiation factor, eIF3, which provides a link to the 40S subunit; and the eIF4E kinases, Mnk1 and Mnk2 (Pyrronet et al. 1999; Mahalingam et al. 2001, Fig. 1). The eIF4E/4G/4A complex is often referred to as eIF4F. Such complexes are thought to be of key importance in mediating normal, cap-dependent, translation initiation. The second group of eIF4E-interacting proteins comprises low molecular mass proteins that bind to the same (or an overlapping) site on eIF4E and block its interaction with eIF4G. In mammals, three 4E-binding proteins are known (4E-BP1/2/3). A third type of partner for eIF4E is the nucleocytoplasmic shuttling protein, 4E-T (Dostie et al. 2000). These proteins (eIF4Gs, 4E-BPs, 4E-T) share a common binding motif through which they interact with eIF4E (Dostie et al. 2000; Haghighat et al. 1995). Binding is therefore mutually exclusive and, for example, eIF4E bound to 4E-BP1 cannot interact with eIF4G to form initiation complexes. 4E-BP1 therefore acts as a repressor of eIF4E-dependent translation.



**Fig. 1** Connections between mTOR and the regulation of translation initiation and elongation factors. mTOR positively regulates the phosphorylation and function of the ribosomal protein (*rp*) S6 kinases and of the eIF4E-binding protein, 4E-BP1. Phosphorylation of S6Ks leads to their activation. S6Ks phosphorylate *rpS6*, which is considered to play a role in the regulation of the translation of the subset of mRNAs containing a 5'-terminal tract of oligopyrimidines (5'-TOP mRNAs). S6Ks also phosphorylate elongation factor 2 (*eEF2*) kinase, leading to a decrease in its activity at basal calcium concentrations. Inactivation of *eEF2* kinase facilitates the dephosphorylation of *eEF2* and the activation of elongation. There appear to be additional mTOR-dependent inputs into the phosphorylation and control of *eEF2* kinase (see text). Phosphorylation of 4E-BP1 at certain sites (see Fig. 2) leads to its release from eIF4E, which can then interact with eIF4G to form initiation complexes and can recruit the 40S ribosomal subunit to the 5'-end of the mRNA. Amino acids and cellular energy act as positive modulators of mTOR. The mechanisms by which amino acids exert this effect are unclear. Insulin and a range of other stimuli increase the phosphorylation of S6Ks and 4E-BP1. However, it is not clear ("??") whether they do so by actually modulating the activity/function of mTOR, or whether they provide separate inputs that nonetheless require the mTOR-dependent input. The TSC1/TSC2 complex may play an important role in linking, e.g. insulin, to regulation of mTOR. The *question mark* by the role of S6 phosphorylation in the translation of 5'-TOP mRNAs denotes the fact that Tang et al. (2001) have recently challenged the prevailing concept that these mRNAs are regulated via S6 kinases/phosphorylation of *rpS6*, at least in response to amino acids. The *question mark* by the role of ATP in regulating mTOR activity (Dennis et al. 2001) is to indicate that recent data also suggest a role for the AMP-activated kinase in regulating mTOR signalling in skeletal muscle (see text; Bolster et al. 2002)





**Fig. 2** Regulation of 4E-BP1. 4E-BP1 contains a binding motif for interaction with eIF4E, and two regulatory domains have also been identified—the RAIP motif towards the N-terminus and the TOS motif at the extreme C-terminus. Seven sites of phosphorylation have been identified. All except S112 (numbering based on human sequence) are Ser-Pro or Thr-Pro sites (Ser112 is followed by Gln). Inhibition of mTOR or amino acid withdrawal results in dephosphorylation of a number of sites in 4E-BP1, especially Ser65 and Thr70 (*underlined*), although Thr37/46 is also affected. Insulin stimulates phosphorylation of Ser65, Thr70, and Ser112, while Ser83/101 appear to be basally phosphorylated. *Unbroken arrows* indicate interplay between sites of phosphorylation that underlies the complex hierarchy of phosphorylation events. For example, phosphorylation at Thr37/46 is required for phosphorylation at Thr70, and phosphorylation at Thr70 and Ser101 is required for phosphorylation at Ser65

Of the three 4E-BPs, 4E-BP1 is easily the most intensively studied and best understood. It undergoes phosphorylation at multiple sites *in vivo*. As indicated in Fig. 2, these sites are located almost throughout its short sequence of approximately 118 amino acids. Phosphorylation shows a marked hierarchy (Gingras et al. 1999, 2001b): phosphorylation of the threonines near the N-terminus is required for modification of Thr70. Phosphorylation at Thr70, and, as we have recently shown, also at Ser101 (Wang et al. 2003), is required for phosphorylation at Ser65. Earlier data suggested that Ser65 and Thr70 were the most important sites for modulating the binding of 4E-BP1 to eIF4E—phosphorylation at Thr70 promotes its release and phosphorylation at Ser65 may prevent rebinding (Fig. 2). Recently, we have shown that S112 also plays a key role in modulating the release of 4E-BP1 from eIF4E (Wang et al. 2003): this appears to be a direct effect, rather than being mediated through an influence on the phosphorylation of Ser65 or Thr70, and agrees with an earlier finding indicating that Ser112 was required for insulin-induced

release of 4E-BP1 from eIF4E (Yang et al. 2000). Phosphorylation of several sites is increased by agents that activate protein synthesis, such as insulin, and this is blocked by rapamycin, indicating an essential role for mTOR in signalling from the insulin-receptor to 4E-BP1 (reviewed in Gingras et al. 2001a). Recent work has identified two regulatory motifs in 4E-BP1, the so-called RAIP motif near its N-terminus (Tee and Proud, 2002) and the C-terminal TOS motif (Schalm and Blenis, 2002), both of which play key roles in the regulation of 4E-BP1 phosphorylation *in vivo*. They may function by binding other regulatory proteins such as molecular scaffolds or kinases that act on 4E-BP1. The reader is referred to the chapters by Gingras et al. and Kim and Sabatini (this volume) for further information on the control of 4E-BP1 and the role of scaffold proteins in mTOR signalling.

## 2.2

### Regulation of 4E-BP1 by Amino Acids

In a number of types of mammalian cells, amino acids exert marked effects on the phosphorylation and regulation of 4E-BP1 (reviewed in Kimball et al. 2000a; Gingras et al. 2001a). For example, when Chinese hamster ovary cells are transferred to a medium lacking amino acids, 4E-BP1 undergoes dephosphorylation, as manifested by a shift to species that migrate faster on SDS-PAGE (Wang et al. 1998; Patel et al. 2001; Hara et al. 1998) which occurs within 15–30 min of amino acid withdrawal. In control cells, in medium containing amino acids, there is little or no 4E-BP1 bound to eIF4E and high levels of eIF4F complexes are present. Removal of amino acids quickly causes a marked increase in the amount of 4E-BP1 associated with eIF4E and loss of eIF4F complexes (Wang et al. 1998; Campbell et al. 1999; Patel et al. 2001). All effects are reversed, within minutes, by re-addition of amino acids. The most effective single amino acid is leucine, others having very little or no effect. This role for leucine is a feature that will be seen again in subsequent discussion. However, even at concentrations well above those present in the cells' normal medium, leucine was unable to induce the level of 4E-BP1 phosphorylation seen in amino acid replete cells, suggesting that the other amino acids are also important in this effect. Indeed, Hara et al. (1998) noted that removal of each of many of the amino acids present in medium affected mTOR signalling, removal of leucine or arginine in-

ducing particularly marked effects on the target of this pathway they studied, S6K1 (see below).

These data show that amino acids themselves, in the absence of hormones such as insulin, have marked effects on the phosphorylation of 4E-BP1 and on the formation of translation initiation factor complexes. They imply that mammalian cells have the ability to sense the prevailing availability of amino acids and to relay this information to the translational machinery.

The situation is subtly different in certain other types of cells: in human embryonic kidney cells, maintained in medium without serum, basal 4E-BP1 phosphorylation is much lower, so that much of the eIF4E is bound to 4E-BP1 and levels of eIF4F complexes are accordingly low (Herbert et al. 2000; Herbert et al. 2002). Upon addition of insulin or the phorbol ester, TPA, 4E-BP1 undergoes phosphorylation leading to its release from eIF4E and formation of eIF4F complexes (Herbert et al. 2000; Herbert et al. 2002). This does not happen in cells maintained in medium lacking amino acids (E. Hajduch and C.G. Proud, unpublished data). In adult cardiomyocytes, there is no such requirement for external amino acids, as insulin can induce phosphorylation of 4E-BP1 in cells kept in amino acid-free medium (L. Wang and C.G. Proud, unpublished data). This issue is discussed in greater detail below.

Insulin elicits an increase in 4E-BP1 phosphorylation in amino acid-replete cells, and can still do so to some extent in CHO cells deprived of amino acids, provided that a metabolisable glucose analogue (or other metabolisable hexose) is also present (Patel et al. 2001). The presence of glucose increases the basal level of phosphorylation of Thr70, but has little effect on basal phosphorylation at Ser65 or Thr37/46. (The reader should note that differences in sequence between 4E-BP1 from human and rat/mouse result in a shift by -1 in the numbering system for all the phosphorylation sites in the rodent polypeptides: the sequence of hamster 4E-BP1 is not known, but the rodent numbering system has been used here for the hamster 4E-BP1.) However, the presence of glucose does allow insulin to elicit phosphorylation at these sites (Patel et al. 2001), thus promoting release of 4E-BP1 from eIF4E and allowing formation of eIF4F complexes. Glucose thus exerts a permissive effect with respect to the action of insulin. This may reflect an input from metabolic energy to the control of 4E-BP1, perhaps via modulation of the activity of mTOR (Dennis et al. 2001). Overall, these data indicate a requirement both for amino acids (especially leucine) and an energy source for acti-

vation of this key step in translation initiation. This clearly makes excellent sense—amino acids are the precursor for protein synthesis, leucine being an essential amino acid, and protein synthesis consumes a large proportion (perhaps 20%–25%; Schmidt 1999) of total cellular energy. We will return to these points later in this chapter.

The group of Kimball and Jefferson has studied in detail the effects of amino acids on 4E-BP1 in isolated adipocytes *in vitro*, in perfused liver, and in skeletal muscle *in vitro* and *in vivo* (reviewed in Kimball et al. 2002; Anthony et al. 2001; Shah et al. 2000). Their data indicate that amino acids exert a positive effect on the phosphorylation of 4E-BP1, with leucine being effective when given alone (Shah et al. 1999; Kimball et al. 2000b; Balage et al. 2001; Pham et al. 2000). In muscle, both insulin and amino acids were required for the ability of insulin to enhance formation of complexes between eIF4E and eIF4G and the rate of protein synthesis. Insulin may serve a permissive function here: for example, giving leucine alone, orally, to rats activates protein synthesis and translation initiation as effectively as a complete meal, but without a rise in plasma insulin concentration (Anthony et al. 2000a). In mice showing defective insulin signalling (with similarities to type II diabetes), feeding still stimulates protein synthesis in a way similar to the effects observed in control animals (Svanberg et al. 1997). However, a marked reduction in circulating insulin levels (achieved using anti-insulin antibodies) does impair the response to leucine (Preedy et al. 1986; Svanberg et al. 1996; Yoshizawa et al. 1995). Taken together, these data indicate that insulin is required for the feeding-induced activation of translation, but that increases in insulin levels may not be, suggesting insulin is playing a permissive role here.

### 3

#### **Regulation of the S6 Kinases**

The protein kinases that phosphorylate ribosomal protein S6 are a second group of proteins that are regulated via mTOR and implicated in the control of mRNA translation (Avruch et al. 2001; Fumagalli et al. 2000; Fig. 1). Through alternative splicing, the S6 kinase 1 ( $\alpha$ ) and 2 ( $\beta$ ) genes give rise to four distinct proteins. S6K1 and S6K2 are activated by all stimuli so far tested (e.g. insulin, growth factors, and phorbol esters) in a rapamycin-sensitive manner (Avruch et al. 2001; Wang et al. 2001; Lee-Fruman et al. 1999). There is evidence that insulin activates S6K1 via PI

3-kinase/PKB signalling, although other studies have provided counter evidence (Dufner et al. 1999), and the molecular mechanisms involved remain to be clarified. In response to certain stimuli (e.g. TPA,  $\alpha_1$ -adren-ergic agonists in cardiac myocytes) activation of S6K1/2 requires sig-nalling via MEK, an upstream activator of the classical MAP kinases or Erks (Wang et al. 2001; Wang and Proud 2002).

As described in detail elsewhere in this volume, activation of the S6Ks involves their phosphorylation at multiple sites in their catalytic domain and in the C-terminal regulatory domain (Avruch et al. 2001; Fumagalli et al. 2000). The protein kinases responsible for phosphorylating the C-terminal sites await conclusive identification, while T229 in the T-loop of the catalytic domain has been shown to be phosphorylated by phospho-inositide-dependent kinase 1 (PDK1) in vitro (Alessi et al. 1998; Pullen et al. 1998). PDK1 is also required for activation of S6K1 in vivo (Williams et al. 2000; Balendran et al. 1999) and for the regulation of a number of other members of the so-called AGC kinase subfamily (Bel-ham et al. 1999; Toker et al. 2000). mTOR has been shown to phosphory-late T389 in vitro, although it is not clear that mTOR is the physiological T389 kinase (for discussion of this, see Avruch et al. 2001). The sensitiv-ity of S6K regulation to rapamycin nevertheless shows that mTOR makes an essential input to the control of the S6Ks.

### 3.1

#### Amino Acids Are Positive Regulators of S6K1

In common with the 4E-BPs, the activity of S6K1 is also sensitive to the nutrient status of the cell (Avruch et al. 2001), although differences are again seen between cell types. In CHO cells, the system with which we have mainly worked, amino acid-replete cells show a substantial basal activity of S6K1 that is further enhanced by the addition of insulin (Wang et al. 1998; Campbell et al. 1999). When cells are transferred to amino acid-free medium, basal activity falls sharply and S6K1 is refrac-tory to stimulation by insulin. Addition of amino acids partially restores both basal activity and insulin-responsiveness, although the addition of both amino acids and glucose is required for substantial recovery of both effects (Wang et al. 1998; Hara et al. 1998; Campbell et al. 1999). This situation shows similarities and differences with respect to that de-scribed above for 4E-BP1/eIF4F in these cells. In both cases, amino acids exert very marked effects in CHO cells; however, whereas amino acids/

glucose suffice for complete formation of eIF4F complexes and to maintain a high level of phosphorylation of phosphorylation of 4E-BP1, full activation of S6K1 requires inputs from both amino acids/glucose and insulin. It is currently unclear whether the requirement, in CHO cells, for both amino acids and an additional input (e.g. from insulin) reflects effects of these agents on different (subsets of) phosphorylation sites in S6K1. In this context it is notable that Hara et al. (1998) reported that addition of high levels of amino acids to CHO-IR cells resulted in as high a degree of activation of S6K1 as was observed with normal levels of amino acids plus insulin. This could suggest that amino acids can elicit the full response if present at sufficiently high levels. This is clearly not true of insulin in cell types such as this, as, in the absence of amino acids, the hormone cannot evoke activation of S6K1 even when added at very high concentrations.

Studies using "real" cells, adipocytes and skeletal muscle, generally reflect the data obtained in other, transformed, cell lines. For example, amino acids stimulate S6K1 in rat adipocytes, and this effect is blocked by rapamycin (Fox et al. 1998a). Orally administered leucine elicits the phosphorylation of S6K1 in skeletal muscle, and this requires insulin, but not an increase in insulin concentration (Anthony et al. 2002). In human forearm muscle, branched-chain amino acids elicit phosphorylation of S6K1 (Liu et al. 2001). These data are largely similar to those discussed above for the regulation of 4E-BP1 by amino acids.

The effects of nutrient stimuli and agents such as insulin are blocked by rapamycin (Shah et al. 2000). In fact, removal of amino acids from CHO cells leads to effects on 4E-BP1 and S6K1 which are qualitatively similar to those of rapamycin treatment. Similar data have been reported for a number of other cell types, including adipocytes and HEK 293 cells (Fox et al. 1998a; Hara et al. 1998), giving rise to the notion that the effects of amino acids are transmitted via mTOR, although there is no formal evidence for this. Evidence in favour of this idea was provided by Hara et al. (1998), who showed that amino acid deprivation led to complete dephosphorylation of S6K1 at T389 (T412 in the numbering system used by these authors), this being a major rapamycin-sensitive and thus mTOR-controlled phosphorylation site in S6K1 (Pearson et al. 1995). Furthermore, and more importantly, they found that a mutant of S6K1 that is resistant to inhibition by rapamycin was also resistant to the effects of amino acid withdrawal. These data suggest that amino acids may signal to S6K1 via mTOR. There is as yet no data on the regulation of

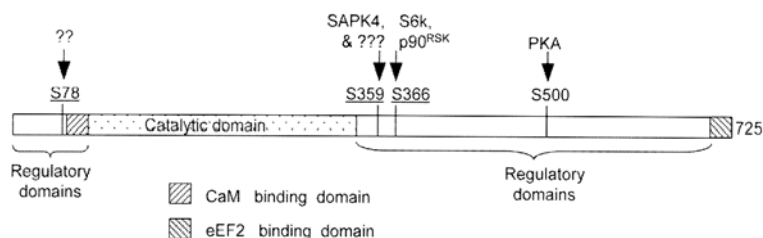
S6K2 by amino acids. However, since, like S6K1, it is regulated via mTOR, it is likely that amino acids also modulate the activity of this enzyme.

One way to assess whether amino acids regulate mTOR itself would be to measure the activity of mTOR extracted (immunopurified) from amino acid-replete or -starved cells. A widely used assay for mTOR relies on its ability, slowly and in the presence of unphysiologically high concentrations of  $Mn^{2+}$ -ions, to phosphorylate 4E-BP1 or S6K1. However, using this assay (with S6K1 as substrate), Dennis et al. (2001) were unable to observe any effect of amino acid withdrawal on the kinase activity of mTOR. Several considerations lead to doubts whether mTOR is the physiological T389 kinase (Avruch et al. 2001).

### 3.2

#### A Role for S6 Phosphorylation in Ribosome Biogenesis?

Work, in particular from the laboratory of George Thomas, has suggested that the S6 kinases may play a role in regulating the translation of a set of mRNAs termed the 5'-TOP (terminal oligopyrimidine tract) mRNAs (Figs. 1 and 3). These mRNAs are characterized by the presence at their extreme 5'-ends of a short sequence of pyrimidines, which represses their translation in serum-deprived cells. Following stimulation of the cells by serum, members of this group of mRNAs (e.g. the mRNA for elongation factor eEF1A) shift into polyribosomes (i.e. initiation onto them is presumably enhanced; Jefferies et al. 1994a, b) leading to increased synthesis of the proteins they encode. These include all the cytoplasmic ribosomal proteins and a number of other components of the translational machinery, e.g. elongation factors eEF1 $\alpha$  and eEF2 (Meyuhas et al. 2000). Amino acids themselves also promote increased synthesis of proteins encoded by 5'-TOP mRNAs such as eEF1A (Kimball et al. 1999) and ribosomal proteins (Anthony et al. 2001). This amino acid- and hormone-regulated translational control mechanism provides a way in which synthesis of components of the translational machinery can be quickly switched on following treatment of mammalian cells by an anabolic/proliferative stimulus to increase the cellular capacity for protein synthesis. Defects in this process may underlie the small size phenotype of cells or animals in which S6K genes have been knocked out (Shima et al. 1998; Montagne et al. 1999).



**Fig. 3** Regulation of eEF2 kinase. The positions of the unusual catalytic domain and the N- and C-terminal regulatory domains. Numbering is based on the human sequence. The N-terminal region contains the binding site for calmodulin (*CaM*) and a phosphorylation site (Ser78), which is regulated by insulin in an mTOR-dependent manner. It is not known which protein kinase is responsible for its phosphorylation. The C-terminus contains three known sites of phosphorylation (Ser359, Ser366, Ser500) and the extreme C-terminal tip is essential for efficient phosphorylation of eEF2. Phosphorylation at Ser359 or Ser366 inactivates eEF2 kinase. Ser366 is phosphorylated by S6Ks and by p90RSK (a downstream effector of the classical MAP/Erk cascade). Although Ser359 is phosphorylated by SAPK4 (p38 MAP kinase  $\delta$ ) in vitro, it is probably not the only kinase acting at this mTOR-regulated site in vivo (see text). Phosphorylation at three sites (*underlined*) is affected by rapamycin in vivo. Ser500 is phosphorylated by cAMP-dependent protein kinase (*PKA*) in vitro and this results in eEF2 kinase becoming partially independent of Ca/*CaM*—essentially, phosphorylation at Ser500 activates eEF2 kinase

The studies of Jefferies and Thomas (Jefferies et al. 1994a, b, 1997) indicated that the translational activation of the 5'-TOP mRNAs was inhibited by rapamycin. The same group subsequently reported that expression of a mutant of S6K1 that is relatively insensitive to rapamycin resulted in decreased sensitivity of 5'-TOP mRNA translation to this drug (Jefferies et al. 1997). Furthermore, disruption of the S6K1 gene interfered with regulation of the translation of ribosomal protein mRNAs. In embryonic stem cells lacking S6K1, serum did not upregulate translation of such mRNAs in the S6K1<sup>-/-</sup> cells, and rapamycin did not affect their association with polysomes either (Kawasome et al. 1998). These data seemed to indicate that S6K1, and perhaps phosphorylation of its best-known substrate, the S6 protein, was involved in activation of 5'-TOP mRNA translation. However, more recent work has challenged this view. Tang et al. (2001) confirmed that 5'-TOP mRNA translation is enhanced by amino acids (which would be consistent with a role for S6K1) but concluded that, by various criteria, S6 phosphorylation did not appear to be sufficient for increased 5'-TOP mRNA translation, at least in



response to amino acids. Their finding that amino acid regulation of 5'-TOP mRNA translation is still observed in cells in which both alleles of the S6K1 gene are knocked out, and no phosphorylation of S6 is observed in response to amino acids, also casts some doubt on the universal role of S6Ks and thus S6 phosphorylation in the control of 5'-TOP mRNA translation by amino acids. Their data do point to a role for signalling via PI 3-kinase in the control of 5'-TOP mRNA translation.

The above findings underline the need for further work to elucidate the mechanisms by which 5'-TOP mRNA translation is controlled and to define the cellular functions of the S6Ks. One such potential function has recently been reported to be in the control of the elongation factor eEF2.

#### 4

### Elongation Factor 2

Elongation factor 2 (eEF2) is a large (ca. 90 kDa) monomer which binds GTP and mediates the translocation step of elongation. This involves the movement of the ribosome by one codon relative to the mRNA and the migration of the tRNAs between sites on the ribosome, e.g. the peptidyl-tRNA moves from the A- into the P-site. eEF2 is a phosphoprotein, the major site of phosphorylation being Thr56 within the GTP-binding site. Phosphorylation of Thr56 prevents eEF2 from binding to the ribosome and thus inactivates it (Carlberg et al. 1990). The kinase responsible for phosphorylation of eEF2 is a calcium/calmodulin-dependent kinase originally referred to as Ca/CaM-kinase III (Nairn et al. 1987; Ryazanov et al. 1988). Since eEF2 is its only known substrate, it is now simply called eEF2 kinase (Fig. 3). Cloning of cDNAs for muscle eEF2 kinase revealed little similarity to the main Ser/Thr/Tyr kinase superfamily or to the mitochondrial/histidine kinases (Redpath et al. 1996b). Ryazanov (Ryazanov et al. 1997, 1999) subsequently noted similarities to a *Dictyostelium* myosin heavy chain kinase and to a number of human ESTs, and coined the term " $\alpha$ -kinases" for this small group of unusual kinases (to reflect the idea—based on the myosin-heavy chain kinase—that they may phosphorylate residues in  $\alpha$ -helical regions of their substrates).

## 4.1

## The Phosphorylation of eEF2 Is Regulated by Insulin and Other Stimuli

In CHO cells overexpressing the insulin receptor, insulin brings about the rapid dephosphorylation of eEF2, concomitantly with accelerating the rate of elongation, and both effects are blocked by rapamycin (Redpath et al. 1996a). Insulin also elicits the dephosphorylation of eEF2 in control CHO cells (Horman et al. 2002), adipocytes (Diggle et al. 1998), mouse embryonic fibroblasts (Wang et al. 2001) and primary cardiomyocytes (Wang et al. 2000). Insulin decreases the activity of eEF2 kinase, and this is also blocked by rapamycin (Redpath et al. 1996a; Wang et al. 2000). These data suggested a link between mTOR and the control of eEF2 kinase. The finding that regulation of eEF2 and of eEF2 kinase is lost in cells lacking PDK1 suggested a role for one or more AGC-family kinases in the regulation of eEF2 kinase. Indeed, eEF2 kinase is a substrate for three such kinases, S6K1 and 2, and the MAP-kinase activated kinase 90<sup>RSK</sup> (Wang et al. 2001). Phosphorylation occurs at a single conserved serine, Ser366 (human sequence, Fig. 3) and results in the inactivation of eEF2 kinase, especially when measured at low (physiological) Ca-ion concentrations (Wang et al. 2001). These findings thus provide a mechanism by which, e.g. insulin, can bring about the dephosphorylation of eEF2 and activate elongation in a rapamycin-sensitive manner (Figs. 1 and 3). They also predict that an alternative regulatory mechanism can also operate, in response to agents that stimulate MAP kinase, through p90<sup>RSK</sup> (Wang et al. 2001). This probably explains how angiotensin II (Everett et al. 2001) and  $\alpha_1$ -adrenergic agonists (L. Wang and C.G. Proud, unpublished data) induce the dephosphorylation of eEF2 in a MEK-dependent manner in cardiomyocytes. One would expect such effects to be independent of mTOR and of external amino acids: this has yet to be tested. Heart cells are not an appropriate system to do this, as they do not require external amino acids for regulation of mTOR signalling (L. Wang and C.G. Proud, unpublished data).

However, other work suggests that additional mTOR-dependent inputs feed into eEF2 kinase. This is implied by the observations that other phosphorylation sites in eEF2 kinase (Fig. 3) are also sensitive to rapamycin. Knebel et al. (2001) showed that phosphorylation of Ser359 is increased by IGF1 and that this effect was blocked by rapamycin. The only kinase known to act at Ser359 is stress-activated protein kinase 4; SAPK4; also called p38 MAP kinase  $\delta$ , an enzyme whose activity is af-

fects neither by IGF1 nor rapamycin. This implies that a further, unknown, IGF-1/mTOR-regulated kinase phosphorylates this site in eEF2 kinase, although it is also possible that, as has been suggested for S6K1 (see this volume), regulation of protein phosphatase activity is involved here too. Phosphorylation of Ser359 decreases the activity of eEF2 kinase (Knebel et al. 2001). More recently, a novel site of phosphorylation has been found near the N-terminal calmodulin-binding motif in eEF2 kinase. Phosphorylation of this residue (Ser78) is increased by insulin and, as for Ser359 and Ser366, this effect is ablated by rapamycin (G.J. Browne and C.G. Proud, unpublished data). The PI 3-kinase inhibitor LY294002 also suppresses the insulin-induced phosphorylation of Ser78 (G.J. Browne and C.G. Proud, unpublished data). The identity of the kinase responsible for phosphorylation of Ser78 is unknown. These data indicate that mTOR regulates the phosphorylation of several sites in eEF2 kinase, two of which act to decrease eEF2 kinase activity, i.e. to promote dephosphorylation and activation of eEF2. The effect of phosphorylation at Ser78 on eEF2 kinase activity is unknown. Its proximity to the calmodulin-binding site (Fig. 3) suggests that phosphorylation here may affect the control of eEF2 kinase by calcium/calmodulin, but in the absence of a kinase with which to modify this site, it is hard to test this directly. It is interesting to note that phosphorylation at Ser366 is already known to decrease the sensitivity of eEF2 kinase to activation by calcium/calmodulin (Wang et al. 2001). The fact that this site lies far from the calmodulin-binding site in the primary sequence of eEF2 kinase suggests that within its tertiary structure, interactions occur between the N- and C-terminal regulatory domains (Fig. 3).

## 4.2

### Nutrients and Cellular Energy Status Modulate the Control of eEF2 Phosphorylation

Given these inputs from mTOR into the control of eEF2 kinase, one might anticipate that the phosphorylation of eEF2 would be modulated by nutrients. Indeed, insulin fails to bring about the dephosphorylation of eEF2 in CHO cells maintained in medium lacking amino acids or glucose (Campbell et al. 1999). Addition of amino acids and glucose to the medium allows insulin again to elicit the dephosphorylation of eEF2. This largely mirrors the behaviour of S6K1 in these cells (Campbell et al. 1999).

A further input from the nutritional status of the cells to the control of eEF2 phosphorylation appears to be related to cellular ATP levels, and may underlie the requirement for glucose referred to above. Treatment of CHO cells or hepatocytes with AICA-riboside leads to markedly increased phosphorylation of eEF2 (Horman et al. 2002). This compound undergoes conversion within the cell to ZMP, which is a specific activator of the AMP-activated protein kinase (AMPK; Hardie et al. 1998). AMPK is stimulated, through several mechanisms, by AMP, levels of which rise as ATP falls. The activity of AMPK is thus increased under conditions of energy deficiency, where the cell needs to shut off nonessential processes that consume energy. The observation that AICAR treatment increases the level of phosphorylation of eEF2 strongly suggests that AMPK makes an input to its control, possibly by activating eEF2 kinase. It clearly makes physiological sense for protein synthesis to be matched to energy availability: protein synthesis is a major energy-consuming process, using up approximately 25%–30% of total cellular energy (Schmidt 1999). To save energy in the short term, it is logical to inactivate eEF2 and thus elongation (where most of the energy is consumed: four high-energy phosphate bonds per amino acyl residue added to the polypeptide chain). Regulation of elongation rather than, e.g. initiation, would have the additional virtue of leaving the polyribosomes intact, rather than causing them to dissociate, obviating the necessity to reform them when energy levels are restored.

Recent data (Bolster et al. 2002) indicate that AMPK may inhibit mTOR signalling in skeletal muscle. Injection of AICAR into rats caused decreased protein synthesis in skeletal muscle and led to the dephosphorylation of both 4E-BP1 and S6K1, suggesting an inhibition of mTOR signalling. A causal relationship between these effects and the inhibition of protein synthesis was not established. It is also possible that the effects are indirect, e.g. they may be mediated by effects of AICAR on other processes or tissues which ultimately impinge on muscle protein synthesis. For example, activation of the AMP-activated kinase may block pancreatic insulin production with secondary effects on translation factor activity in muscle (da Silva et al. 2000). Nevertheless, the idea that AMPK acts to inhibit protein synthesis is an attractive one given the role this kinase plays in controlling multiple facets of energy metabolism (Hardie et al. 1998). The roles in this for general effects on mTOR signalling vs. specific regulation of eEF2 phosphorylation require further study.

mTOR shows a relatively very high  $K_m$  for ATP, so that a fall in cellular ATP will decrease its activity (Dennis et al. 2001). The effect of AICAR on eEF2 phosphorylation does not reflect the operation of this effect, since (1) AICAR does not decrease cellular ATP levels and (2) AICAR does not significantly affect the phosphorylation of other targets of mTOR signalling such as 4E-BP1 and S6K1 (Horman et al. 2002) in CHO cells. Treatment of cells with 2-deoxyglucose depletes ATP (as this sugar is a substrate for phosphorylation to the 6-phosphate, thus using up ATP, but cannot be fully metabolised through glycolysis, and so does not yield any ATP). Low (<10 mM) concentrations of 2-deoxyglucose cause a maximal increase in the phosphorylation of eEF2, again without affecting other targets for mTOR. This is consistent with the effects of mild ATP depletion on eEF2 phosphorylation being mediated via the AMPK rather than through inhibition of mTOR (Dennis et al. 2001; Horman et al. 2002). High concentrations of 2-deoxyglucose impose a much more severe metabolic stress and are accompanied by inhibition of S6K1 and dephosphorylation of 4E-BP1 (Dennis et al. 2001; Horman et al. 2002). However, this does not cause a further increase in eEF2 phosphorylation, which is, as stated above, already maximal.

Similarly, in adult rat cardiomyocytes, AICAR causes a marked rise in the phosphorylation of eEF2 without significant effects on 4E-BP1, again suggesting that activation of AMPK affects eEF2 phosphorylation independently of mTOR. In contrast, agents which exert large effects on ATP levels by interfering with its synthesis (such as uncouplers or oligomycin) do lead to dephosphorylation of 4E-BP1 (perhaps due to impaired mTOR signalling) but do not increase eEF2 phosphorylation to a greater extent than AICAR does (L.E. McLeod and C.G. Proud, unpublished data). Thus, eEF2 phosphorylation may be particularly sensitive to small decreases in ATP levels, while more drastic falls in ATP act to impair mTOR activity and interfere with translation initiation, by causing inhibition of eIF4E.

In summary, the phosphorylation of eEF2 increases in response to relatively mild energy depletion, presumably leading to a slow down or pausing of elongation. Physiologically, this is presumably reversed when cellular energy levels recover, allowing a resumption of translation. More severe energy challenges cause a greater depletion of ATP, leading to decreased activity of mTOR and the dephosphorylation of S6K1 and 4E-BP1, with effects, one assumes, on cap-dependent translation and probably the translation of 5'-TOP mRNAs. These effects are likely to be of

major importance in matching protein synthesis to the energy and metabolic status of the cell, and likely come into play physiologically and under pathological conditions such as hypoxia and ischemia, e.g. as a consequence of stroke.

In *Saccharomyces cerevisiae*, glucose starvation leads to a rapid and reversible inhibition of protein synthesis (Ashe et al. 2000), which is associated with loss of polyribosomes. This is indicative of inhibition of translation initiation rather than elongation. However, this effect is still observed in a yeast strain that is resistant to rapamycin, due to a mutation in the phosphatase-binding protein Tap42 (Di Como et al. 1996). Thus, the effect of glucose withdrawal in yeast appears unlikely to be mediated through the TOR proteins. In contrast, a strain in which the genes for the two glucose sensors Snf3 and Rgt2 (Ozcan et al. 1998) are deleted does show resistance to glucose depletion, suggesting that they are involved in relaying information about prevailing glucose levels to the translational machinery. It is currently unclear which components of the initiation machinery are regulated during this response.

## 5

### **Contribution of mTOR Signalling to the Regulation of Protein Synthesis In Vivo**

An important question is, to what extent do the above regulatory events, linked to mTOR signalling, contribute to the activation of protein synthesis in cells and tissues? This question can be addressed by exploring the effect of rapamycin on the control of overall rates of protein synthesis. In cell lines, rapamycin generally exerts only a small inhibitory effect on the rate of protein synthesis (see, e.g., Beretta et al. 1996).

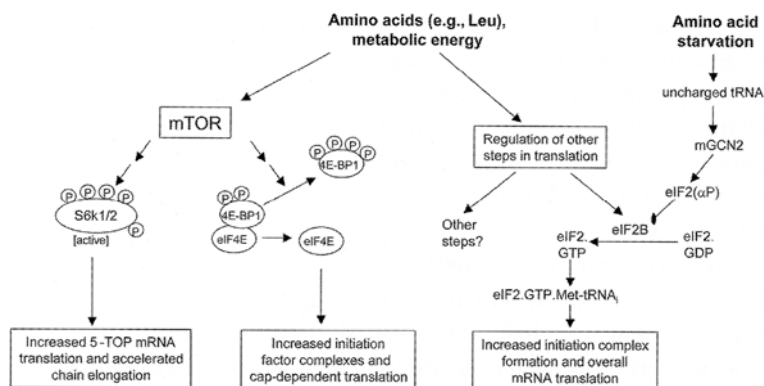
In skeletal muscle, the activation of protein synthesis elicited by leucine is only partially blocked by rapamycin (Anthony et al. 2000b). This suggests that leucine may operate to stimulate protein synthesis via both mTOR-dependent and -independent pathways. Indeed, leucine is still able to activate muscle protein synthesis in alloxan-diabetic rats, where there is no S6K1 phosphorylation or eIF4E/eIF4G binding in response to oral leucine (Anthony et al. 2002). This points to the operation of perhaps two amino acid-regulated responses: firstly, regulation of proteins, such as S6K1 and 4E-BP1, which are linked to mTOR and which require insulin, and, secondly, an insulin-independent pathway which does not impact on these two proteins and may therefore be distinct from events

linked to mTOR (and is therefore insensitive to rapamycin). Additional, leucine-sensitive, regulatory inputs must therefore also operate to control protein synthesis in skeletal muscle. Indeed, in L6 myoblasts, eIF2B (a translation factor not linked to mTOR signalling) appears to be of more importance than the control of eIF4E (by 4E-BP1) for the activation of protein synthesis by amino acids (Kimball et al. 1998). In isolated rat heart cells (ventricular myocytes), protein synthesis is acutely activated by insulin (Wang et al. 2000) or  $\alpha_1$ -adrenergic agonists (Wang et al. 2002) and these effects are blocked by approximately 50% by rapamycin. This indicates that mTOR signalling does play an important role in the activation of protein synthesis by these stimuli, but again underlines the point that other regulatory mechanisms are also important.

## 6

### Other Targets for Control by Nutrients

eIF2B is a multisubunit protein that mediates nucleotide-exchange on eIF2, the translation initiation factor that recruits the initiator methionyl-tRNA to the 40S subunit to recognize the start codon during translation initiation (Hinnebusch 2000) (Fig. 4). The activity of eIF2B plays a role in regulating overall and transcript specific translational control in eukaryotes from yeast to mammals, and is regulated by a variety of inputs (Hinnebusch 2000). It can be regulated by amino acids, apparently via several distinct mechanisms, although these do not appear to involve signalling via mTOR. For example, rapamycin does not affect the ability of insulin to activate eIF2B in CHO.T cells (Welsh et al. 1997). Activation of eIF2B in these cells requires the presence of amino acids and glucose in the medium (Campbell et al. 1999). These effects do not appear to be connected with defects in the ability of insulin to promote the dephosphorylation of a regulatory (inhibitory) phosphorylation site in the  $\epsilon$ -subunit of eIF2B (which still occurred in the absence of glucose or amino acids; Campbell et al. 1999). In yeast, amino acids regulate the phosphorylation of the  $\alpha$ -subunit of eIF2, via the kinase GCN2 (Fig. 4). Hence, during amino acid starvation phosphorylation is increased, giving rise to eIF2( $\alpha$ P), which potentially inhibits eIF2B. Such a mechanism does not seem to be involved in the effects reported by Campbell et al. (1999), as amino acid withdrawal does not affect eIF2 $\alpha$  phosphorylation in CHO cells (Campbell et al. 1999; Patel et al. 2001). In L6 myoblasts, histidine and leucine were each able to activate eIF2B and total protein



**Fig. 4** Nutrient inputs into the control of translation in mammalian cells. Data from studies, e.g. in skeletal muscle, suggest that nutrient regulation of protein synthesis (e.g. in response to administration of leucine) does involve mTOR signalling (*left side*) but that additional events are also involved. These may include modulation of the guanine nucleotide exchange factor eIF2B and perhaps other unknown events (*dotted line*). Several studies indicate that amino acids (in some cases in combination with a source of metabolic energy, glucose) activate eIF2B or modulate its control by insulin (see text). The mechanisms involved are unclear. One potential mechanism for its control by amino acids could involve the eIF2 kinase, mGCN2. This enzyme is thought to be activated by uncharged tRNA and phosphorylates eIF2, to create a competitive inhibitor of eIF2B. However, this apparently cannot explain the regulation of eIF2B amino acids that is observed in several studies using mammalian cells, since eIF2 $\alpha$  phosphorylation did not change, and the role that mGCN2 plays in these responses (if any) remains unclear

synthesis, while only leucine was able to modulate 4E-BP1, via the mTOR pathway (Kimball et al. 1998, 1999). These data also imply that regulation of the activity of eIF2B, rather than the control of 4E-BP1 (or by implication, S6K1) is important for the overall regulation of protein synthesis in these cells. Control of 4E-BP1 and S6K1, via mTOR, in response to leucine, appears rather to control the translation of specific mRNAs (Kimball et al. 1999). Changes in the activity of eIF2B are also implicated in the overall control of protein synthesis in the liver in response to amino acid imbalance (elevated levels of leucine, glutamine, and tyrosine) on the basis of correlations between eIF2B activity (but not levels of eIF4E, for example) and overall protein synthetic rates (Shah et al. 1999). The molecular mechanisms involved here are unclear, but may involve changes in the activity of a protein kinase that phosphorylates the catalytic  $\epsilon$ -subunit of eIF2B and shows decreased activity in



response to amino acid imbalance. The activity of this unidentified kinase was not affected by rapamycin pre-treatment of the cells, again indicating that mTOR is not involved here (Shah et al. 1999).

## 7

### **Do Mammalian Cells Sense Intracellular or Extracellular Amino Acids?**

#### 7.1

##### **Branched Chain Amino Acids and mTOR Signalling**

Leucine appears to be the only amino acid capable of eliciting an effect on 4E-BP1 and S6K1 in skeletal muscle (Anthony et al. 2000a, b; Vary et al. 1999), while other branched-chain amino acids (isoleucine, valine) are also effective in liver (Anthony et al. 2001; Kimball et al. 2002). In CHO cells, leucine was the only one of the amino acids tested which, when added alone, stimulated 4E-BP1 phosphorylation (Wang et al. 1998), and, conversely, leucine was also the amino acid, which when omitted, had the most profound effect on the activity of S6K1 (Hara et al. 1998). Omission of arginine also resulted in a marked fall in S6K1 activity. It seems likely that cell types differ in their sensitivities to the omission or addition of specific amino acids. Whether this reflects the operation of different sensing mechanisms awaits further information on the sensing process itself.

#### 7.2

##### **How Are Amino Acids Sensed in Animal Cells?**

This raises the key issue of the mechanism by which amino acids are sensed by mammalian cells. Little information is currently available on this. Iiboshi et al. (1999) published information suggesting that levels of tRNA charging may underlie the control of the mTOR pathway. A similar mechanism underlies the control of the phosphorylation of the  $\alpha$ -subunit of eIF2: this has been most intensively studied in yeast, where amino acid starvation leads to increased phosphorylation of eIF2 $\alpha$  and, because eIF2[ $\alpha$ P] is a potent inhibitor of eIF2B, to inhibition of overall protein synthesis (Hinnebusch 2000). The effect involves the activation of the eIF2 $\alpha$  kinase, Gcn2p, by uncharged tRNA (Dong et al. 2000; see Fig. 4). Gcn2 homologues are now known from metazoans (Sood et al. 2000; Berlanga et al. 1999) and may conceivably play similar roles in

controlling protein synthesis under certain conditions. However, the importance of this mechanism in the short-term effects of leucine and other amino acids on signalling through the mTOR pathway is questionable. As mentioned above, we have consistently been unable to see any change in the state of phosphorylation of eIF2 $\alpha$  in response to amino acid addition or withdrawal in CHO cells (Campbell et al. 1999; Patel et al. 2001). Only very small changes in eIF2 $\alpha$  phosphorylation were seen upon leucine deprivation in L6 myoblasts (Kimball et al. 1998) and Dennis et al. (2001) saw no effect of amino acid withdrawal on the level of charging of tRNA. Amino acid alcohols can inhibit amino acyl-tRNA synthetases and thus block tRNA charging. Iiboshi et al. (1999) reported that treatment of T-lymphoblastoid (Jurkat) cells with amino acid alcohols led to decreased S6K1 activity. In contrast, neither we (Patel et al. 2001; L. Wang, A. Beugnet, and C.G. Proud, unpublished data) nor others (Pham et al. 2000) have observed effects of amino acid alcohols on targets of mTOR signalling. Furthermore, inhibition of hepatic protein synthesis by low amino acid levels seems independent of tRNA (Flaim et al. 1982).

How then are amino acids sensed? Amino acids also regulate (repress) autophagy, e.g. in the liver, and this has prompted a number of studies into the mechanisms involved in this effect. By-products of leucine metabolism seem unlikely to be involved here (see discussion of Shah et al. 1999), suggesting a role for leucine itself. Mortimore and colleagues (Mortimore et al. 1994; Miotto et al. 1992) were able to show that a non-cell permeant leucine “analogue” could still inhibit autophagy, implying that the effect was mediated by extracellular leucine, and went on to use photoaffinity labelling to show that this reagent could label a membrane-associated protein of 340 kDa, suggesting the existence of a plasma membrane leucine “sensor” (Miotto et al. 1992). This idea is attractive especially in view of the discovery of a plasma membrane amino acid sensor (Ssy1p) in yeast (Iraqi et al. 1999), but the molecular identity of this protein in mammalian cells has not been established.

In the case of the effects of leucine on mTOR signalling, various structural homologues of leucine can also mediate the effect (see Shah et al. 1999; Patti et al. 1998), but other evidence suggests that leucine, rather than its immediate transamination product  $\alpha$ -ketoisocaproate, is the mediator of the effect (Fox et al. 1998b). The specificity for leucine may vary between cell types: while it is the primary regulator in adipocytes, this is not universally the case (Lynch 2001). Deprivation of amino acids caused a marked depletion of branched chain amino acids (Dennis et al.

2001), raising the alternative possibility that vertebrate cells respond to intracellular amino acid levels. Other data consistent with this idea are the observations (1) that injection of leucine into *Xenopus* oocytes activated TOR signalling (specifically, the phosphorylation and activity of S6k (Christie et al. 2002); and (2) the finding that manipulation designed to alter intracellular amino acid levels affect mTOR signalling. For example, blocking intracellular proteolysis (autophagy) renders hepatoma cells dependent upon added, external amino acids, perhaps by decreasing the pool of intracellular amino acids (Shigemitsu et al. 1999). Conversely, it is well known that cycloheximide, an inhibitor of protein synthesis, activates S6K1 (Price et al. 1989). Our recent studies have shown that this compound, and several other inhibitors of protein synthesis, also enhance the phosphorylation of 4E-BP1 (Beugnet et al. 2003) and promote the formation of eIF4F complexes, as well as the phosphorylation and activation of S6K1 and the phosphorylation of its intracellular substrate ribosomal protein S6. These effects may reflect the ability of these compounds to increase intracellular amino acid levels by blocking their use in protein synthesis. This idea is supported by the observations that protein synthesis inhibitors do indeed increase the level of intracellular amino acids in amino acid-starved cells, that their effects persist when the medium is changed for fresh medium devoid of amino acids, and that their positive effect on 4E-BP1 and S6K1 is impaired by blocking protein breakdown, which is expected to decrease intracellular amino acid levels (Beugnet et al. 2003). Thus, mammalian cells may possess an intracellular amino acid coupled to mTOR signalling. This seems likely to be selective for branched chain amino acids, in particular leucine (although some studies suggest arginine may also be particularly effective)..

## 8

### Future Directions

The last 4 years or so have seen important advances in our understanding of the control of translation factors by nutrients. However, as is so often the case, the recent data raise even more questions. Particularly important issues concerning the role of mTOR signalling in coupling amino acid availability to the control of translation factors include: the nature of the machinery by which amino acids are sensed in mammalian cells, and the mechanisms by which this information is relayed to

mTOR, and the links between mTOR and the control of the S6Ks and 4E-BPs. For example, it is important to identify the protein kinases acting on 4E-BP1 and the S6Ks. The complex hierarchy of phosphorylation of 4E-BP1, in particular, suggests that multiple kinases are involved, some of which may be basally active (due to an input from mTOR), while others may be turned on by insulin. The role of regulation of protein phosphatases in the control of 4E-BPs and S6Ks also needs to be explored. In a wider context, it is crucial to identify the other regulatory mechanisms, independent of mTOR, that activate protein synthesis in muscle, for example, in response to feeding.

*Acknowledgements.* Work in the author's laboratory is supported by the Biotechnology and Biological Sciences Research Council, the British Heart Foundation, The European Union, The Medical Research Council and the Wellcome Trust.

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## 1

**Introduction**

The potent immunosuppressive, antifungal, and potential antitumor activities of the bacterial macrolide rapamycin derive from its inhibitory action on cell growth and proliferation in organisms ranging from yeast to human. In complex with its cellular receptor FKBP12, rapamycin binds and inhibits the target of rapamycin (TOR), conserved in yeast, flies, worms, and mammals. The TOR proteins belong to the family of phosphatidylinositol kinase-like kinases (PIKK; Keith and Schreiber 1995), which is a unique family of serine/threonine kinases. Tor1p and Tor2p in *S. cerevisiae* control a wide range of growth-related cellular processes, including transcription, translation, and reorganization of the actin cytoskeleton (reviewed in Schmelzle and Hall 2000). The mammalian TOR (mTOR; also named FRAP or RAFT1; Brown et al. 1994; Chiu et al. 1994; Sabatini et al. 1994; Sabers et al. 1995) is likely to be similarly pleiotropic and essential in regulating mammalian cellular functions, although some of the biochemical pathways may be distinct from those in yeast.

The best known function of mTOR in the context of cell proliferation is regulation of translation initiation (recently reviewed in Gingras et al. 2001), mostly mediated by the ribosomal subunit S6 kinase 1 (S6K1) and the eukaryotic initiation factor 4E binding protein 1 (4E-BP1), both regulators of mitogen-induced translation initiation (reviewed in Fumagalli and Thomas 2000; Gingras et al. 1999). Both activation of S6K1 and phosphorylation of 4E-BP1 are stimulated by mitogens and inhibited by rapamycin, and the kinase activity of mTOR is required, but not sufficient, for this regulation (Brown et al. 1995; Brunn et al. 1997). In addition to cell proliferation, mTOR has also been implicated in regulation of apoptosis, cell survival, and differentiation. The biochemical mechanisms of mTOR signaling have been a topic of intense research interest. In this chapter, some recent findings from our laboratory are discussed which have uncovered new components of the mTOR pathway and novel modes of regulation.

## 2

**Direct Interaction of Phosphatidic Acid and the FKBP12-Rapamycin Binding Domain of mTOR**

Located immediately N-terminal to the sequence-defined kinase domain, the FKBP12-rapamycin binding (FRB) domain in mTOR spans amino acids 2,025–2,114 (Chen et al. 1995), and forms a four-helical bundle as revealed by crystal structural analysis (Choi et al. 1996). A regulatory role has been intuitively assigned to FRB, but the mechanism for rapamycin inhibition of mTOR has not been clear (discussed in Gingras et al. 2001). A drastic dominant negative effect of FRB on the G1 cell cycle progression was observed when purified FRB was microinjected into human osteosarcoma MG63 cells (Vilella-Bach et al. 1999). In search for a putative regulator interacting with the FRB domain, we have considered lipid molecules as candidates since FRB's helical bundle structure is reminiscent of many apolipoproteins (Segrest et al. 1992), and found that FRB binds phosphatidic acid (PA)-containing small unilamellar vesicles with striking selectivity (Fang et al. 2001). Preincubation with the rapamycin-FKBP12 complex abolishes FRB's ability to bind PA (Fang et al. 2001), consistent with the possibility that rapamycin may compete with PA for binding to FRB. Identification of an arginine residue (2109) on FRB critical for its electrostatic interaction with PA has established a close correlation between FRB-PA binding and mTOR signaling (Fang et al. 2001). mTOR thus may be added to a growing list of PA-regulated proteins, including several protein kinases and phosphatases (reviewed in English et al. 1996). It should be noted that *in vivo* association between mTOR and PA has yet to be demonstrated, although an interaction between the full-length mTOR protein and a biotinylated PA has been observed *in vitro* (Y. Fang, L. Qian, G. Prestwich, and J. Chen, unpublished observation).

A large fraction of cellular mTOR appears to associate with intracellular membranes (Sabatini et al. 1999; M. Vilella-Bach, R. Bachman, J. Chen, unpublished data; Withers et al. 1997), although the nature of the membrane structure is unknown. The discovery of PA association with the FRB domain in mTOR led to speculation that PA might serve to anchor mTOR in the membrane. This possibility, however, has been ruled out by the observation that a mutant mTOR with the FRB domain deleted associates with cellular membranes to the same extent as wild-type mTOR (M. Vilella-Bach, R. Bachman, J. Chen, unpublished data). In fact,

several regions within the N-terminal two thirds of mTOR display redundant ability in targeting mTOR to membranes (M. Vilella-Bach, R. Bachman, J. Chen, unpublished data), similar to the observation that putative HEAT motifs in the N-terminal region of Tor2p are capable of anchoring the protein in the plasma membrane (Kunz et al. 2000). It thus appears that PA may regulate mTOR signaling by a mechanism other than controlling the membrane association of mTOR.

### 3

#### **Phosphatidic Acid-Mediated Mitogenic Activation of mTOR Signaling**

PA has emerged in recent years as an important lipid second messenger participating in a variety of intracellular signaling events, and it can function as a mitogen in some cell types (English et al. 1996). The normally low concentrations of PA in cellular membranes increase as a result of mitogenic actions of various growth factors and hormones, most likely through phospholipase D (PLD) activation (reviewed in English et al. 1996; Exton 1999). We have indeed observed a rapid increase of PA levels upon serum stimulation of human embryonic kidney (HEK) 293 cells. The increase of PA is abolished by a low concentration of 1-butanol (Fang et al. 2001), which blocks PLD-catalyzed conversion of phosphatidylcholine (PC) to PA by forming phosphatidylbutanol (Yang et al. 1967). In the same cells, butanol also inhibits serum-stimulated S6K1 activation and 4E-BP1 phosphorylation (Fang et al. 2001). The butanol effect appears specific for the mTOR pathway, as the serum-activation of MAP kinase (ERK1/ERK2) is not affected, nor is Akt (Fang et al. 2001). Similarly, 1-butanol inhibits lysophosphatidic acid (LPA)-activated S6K1 in Swiss3T3 cells (Willard et al. 2001). The notion that PA may be a critical component of the mTOR pathway is further supported by the finding that exogenous PA stimulates S6K1 activation and 4E-BP1 phosphorylation in the absence of mitogens (Fang et al. 2001).

Mitogenic stimulation of S6K1 activation and 4E-BP1 phosphorylation is dependent on amino acid sufficiency in the cell (Hara et al. 1998; Iiboshi et al. 1999; Xu et al. 1998). While phosphatidylinositol-3-kinase (PI3K) is essential in relaying mitogenic signals to S6K1 and 4E-BP1 (reviewed in Fumagalli and Thomas 2000; Gingras et al. 2001), mTOR is believed to govern the amino acid-sensing pathway (Hara et al. 1998), although the exact sensing mechanism is still unknown. The discovery of PA as a regulator of mTOR signaling has established a direct link be-

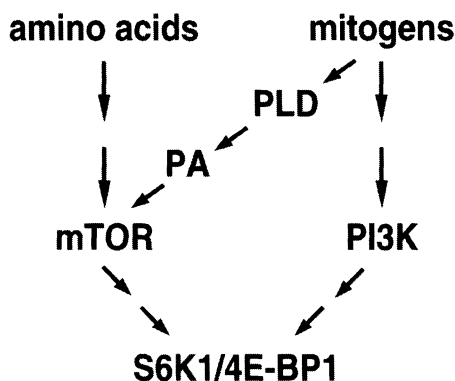
tween mitogens and mTOR, and revealed mTOR's role to integrate mitogen and nutrient signals.

What would be the molecular consequence of PA-mTOR interaction? Neither PA nor butanol has any effect on the kinase activity of mTOR *in vitro* or *in vivo* (Y. Fang and J. Chen, unpublished data). In addition, mTOR mutants that have diminished affinity for PA display wild-type kinase activity (Fang et al. 2001). Thus, PA does not appear to regulate mTOR catalytic activity, which supports the notion that rapamycin does not inhibit mTOR kinase activity. It is not clear exactly how PA regulates mTOR's signaling activity, but several possibilities exist. For instance, PA may help recruit a downstream effector, remove a negative regulator, or modulate mTOR kinase specificity toward a physiological substrate. In any case, the mutually exclusive interactions of FRB-PA and FRB-rapamycin suggest that rapamycin likely exerts its inhibitory effect by blocking PA binding to mTOR *in vivo*. It remains to be seen whether this role of PA is specific for mammals or universal in eukaryotes.

#### 4

### **Three Pathways Are Required in Parallel for mTOR Downstream Signaling**

What is the relationship between PA and the two previously characterized pathways of S6K1 activation/4E-BP1 phosphorylation—the PI3K pathway and the amino acid-sensing mTOR pathway? Amino acid withdrawal abolishes PA's ability to induce S6K1 activation and 4E-BP1 phosphorylation (Fang et al. 2001), suggesting that PA may not lie directly in the amino acid-sensing pathway. Although PA can mimic mitogens in activation of mTOR downstream signaling, PI3K is not activated in PA-treated cells (Fang et al. 2001). In addition, serum activation of a rapamycin-insensitive/wortmannin-sensitive mutant S6K1 is resistant to butanol treatment (Fang et al. 2001), further supporting the notion that PA and PI3K lie on separate pathways. Nonetheless, wortmannin blocks PA's action, suggesting that the basal activity of PI3K is required for PA to stimulate S6K1 activation and 4E-BP1 phosphorylation. Taken together, the simplest model explaining these observations is one where three pathways integrate their signals on S6K1/4E-BP1: an amino acid-sensing mTOR pathway, a mitogen-activated PI3K pathway, and a mitogen-activated PA-mTOR pathway (likely mediated by PLD; Fig. 1).



**Fig. 1** Regulation of S6K1 and 4E-BP1 requires three pathways in parallel (see text)

5

### Candidates for Upstream Regulators of mTOR

The emergence of PA as an activator of mTOR, together with butanol's inhibitory effect, suggests a critical involvement of PLD in the mTOR pathway. When coexpressed with S6K1, a catalytically inactive PLD1 (Sung et al. 1997) acts as a dominant negative mutant by inhibiting mitogenic stimulation of S6K1 activity (Y. Fang and J. Chen, unpublished data), providing further evidence that PLD1 may indeed be an upstream component of the mTOR pathway. Regulation of PLD1 has been found to involve three upstream regulators: the conventional protein kinase C (cPKC), and the small G proteins ARF and Rho (reviewed in Exton 1999; Frohman et al. 1999). Interestingly, some of the previous observations reported in the literature or made by our laboratory may receive new interpretations in light of the novel PLD-PA-mTOR pathway. For instance, constitutively active Cdc42 and Rac1 have been found to activate S6K1 in vivo (Chou and Blenis 1996). The activated Rho proteins can bind, but do not activate S6K1 in vitro (Chou and Blenis 1996). It has thus been proposed that the membrane recruitment of S6K1 via Cdc42 or Rac1 may facilitate S6K1 activation by a membrane-bound kinase, such as PDK1 (Alessi et al. 1998; Peterson and Schreiber 1998). However, activation of PLD1, and consequently mTOR, may provide an alternative mechanism for the Rho proteins' action on S6K1.



The involvement of cPKC in the S6K1 pathway is implicated by the inhibitory effect of a specific PKC $\alpha/\beta$  inhibitor on serum-stimulated S6K1 activation in HEK293 cells (Y. Fang and J. Chen, unpublished data). Although a kinase-independent function of PKC appears to activate PLD1, the kinase activity of PKC has also been linked to the regulation of PLD1 (Houle and Bourgoin 1999). PLD1 and PA could potentially transduce signals from cPKC to S6K1 in vivo. Another set of observations that has not been previously explained is the participation of cellular calcium in activation of S6K1. In liver epithelial cells, angiotensin II-induced S6K1 activation seems to require calcium (Graves et al. 1997), and a cell-permeable Ca<sup>2+</sup> chelator blocks LPA- and serum-stimulation of S6K1 in Swiss3T3 and HEK293 cells, respectively (Y. Fang and J. Chen, unpublished data). These calcium effects may again be attributed to PLD1, since PLD1 activation has been reported to require calcium (Exton 1999), although whether cPKC or other regulators mediates the calcium action remains to be determined. Alternatively, calcium clustering of PA (Buckland and Wilton 2000) may effectively raise the local concentration of PA, which in turn activates mTOR. The array of PLD1 mutants (Du et al. 2000; Zhang et al. 1999) displaying selective response to upstream regulators should prove powerful in delineating the roles of PLD1, cPKC, Rho, and ARF in the PA-mTOR pathway.

Although PLD appears to be the enzyme largely responsible for mitogen-stimulated PA in mTOR signal transduction in HEK293 cells, it is conceivable that some cell types may use alternative routes for PA synthesis. For instance, diacylglycerol (DAG) kinase (DGK) converts DAG to PA. In HEK293 cells, DGK does not seem to contribute to mTOR signaling, as a DGK inhibitor has no effect on S6K1 activation (Y. Fang and J. Chen, unpublished data). However, it cannot be ruled out that some of the nine mammalian DGKs with distinct cell and tissue distributions (van Blitterswijk and Houssa 2000) may influence the mTOR pathway in certain cell types. Another potentially relevant enzyme is lysophosphatidic acid acyltransferase (LPAAT), which catalyzes the conversion of LPA to PA. Although it is not clear how LPAAT activity may be regulated, increased LPAAT- $\beta$  expression levels have been found to associate with many human tumors (Leung 2001), suggesting that LPAAT- $\alpha$  may be involved in mitogenesis and oncogenesis. A potential link between LPAAT and mTOR signaling should be probed in future studies.

## 6

**Cytoplasmic-Nuclear Shuttling of mTOR**

Of all the PIKK family members, the TOR proteins were thought to be the only ones not residing or functioning in the nucleus. However, recent observations suggest otherwise, at least in the case of mTOR. A small fraction of mTOR has been consistently found in the nucleus in cell fractionation experiments (Kim and Chen 2000). Initially thought to be an experimental artifact, the nuclear residence, and furthermore cytoplasmic-nuclear shuttling, of mTOR has been confirmed by its accumulation in the nucleus upon treatment by leptomycin B (LMB; Kim and Chen 2000), a specific inhibitor of the nuclear export receptor Crm-1 (Nishi et al. 1994). The subcellular distribution and rate of cytoplasmic-nuclear shuttling of mTOR are not regulated by known upstream signals, and they vary among different cell types (R. Bachman, J. Kim, J. Chen, unpublished data), possibly due to variations in the availability of the nuclear transporting machinery utilized by mTOR. Classic nuclear localization sequences have not been found in mTOR, implying that an unconventional mechanism may be responsible for the nuclear import of this protein. On the other hand, Rev-like nuclear export signal (NES) sequences are identified in mTOR, which can mediate nuclear export of reporter proteins (R. Bachman, J. Kim, J. Chen, unpublished data).

The first hint that cytoplasmic-nuclear shuttling of mTOR may be functionally important came from the observation that LMB inhibited S6K1 activation and 4E-BP1 phosphorylation (Kim and Chen 2000). Such an inhibition could be simply explained by a depletion of cytoplasmic mTOR due to nuclear sequestration by LMB. However, mTOR with enhanced nuclear localization ability by tagging of the SV40 nuclear localization signal (NLS) displays *increased* signaling activity toward S6K1 and 4E-BP1, whereas mTOR tagged with the HIV Rev NES has weakened signaling ability (Kim and Chen 2000). Taken together, these observations suggest that nuclear entry, as well as nuclear exit, may be crucial for mTOR's function in the cytoplasm. In other words, mTOR needs to gain a nuclear experience before activating the translational machinery (S61, 4E-BP1) and subsequently translation initiation (Kim and Chen 2000). To maximize the effect of such a nuclear experience on cytoplasmic functions, an optimal shuttling rate may be required for mTOR. Consistent with this notion, a single NLS tagged to mTOR enhanced its signaling activity, whereas additional NLS tagging (e.g., four copies) re-

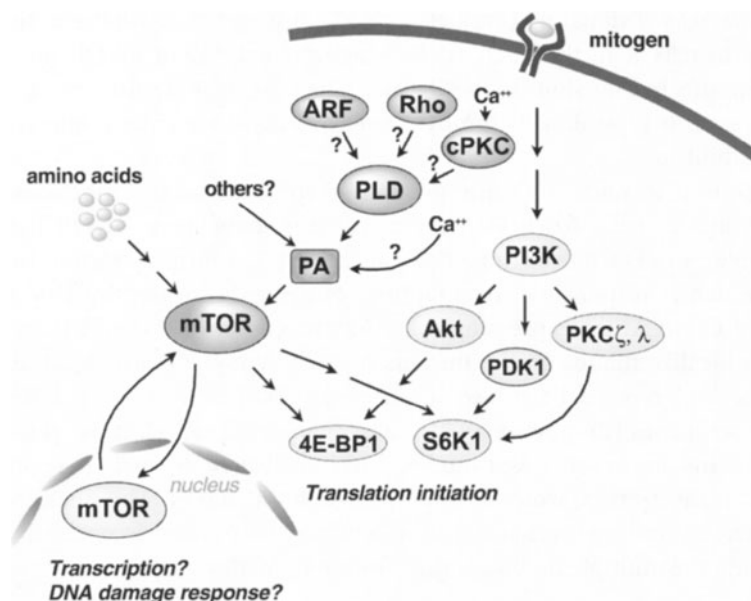
sults in *decreased* signaling (Park et al. 2002). It is not clear what exactly happens to mTOR in the nucleus. The catalytic activity of mTOR does not seem affected by shuttling (Kim and Chen 2000). Recruitment of a cofactor and/or phosphorylation by a nuclear kinase are among numerous possibilities.

In addition to nuclear shuttling required for cytoplasmic functions, distinct nuclear roles for mTOR are possible. For instance, mTOR has been shown to associate with p53 and mediate p53 phosphorylation on Ser15, which is responsible for apoptosis of syncytia formed by HIV-1 Env- and CD4/CXCR4-expressing cells (Castedo et al. 2001). mTOR appears to localize mainly in the nucleus of such syncytia (Castedo et al. 2001). Several reports have also implicated mTOR and S6K1 in DNA damage responses (Brenneisen et al. 2000; Zhang et al. 2001). If the yeast TOR proteins are to serve as a model, other nuclear roles, such as regulation of transcription, would also be plausible for mTOR. The nuclear-cytoplasmic shuttling mechanism of mTOR may be devised to optimally coordinate the multiple pathways this protein regulates.

## 7

### Concluding Remarks

The identification of PA as a second messenger in mTOR regulation has uncovered a new pathway that connects mitogenic signals to mTOR. Thus, mTOR integrates both nutrient and mitogen signals to regulate the translational modulators S6K1 and 4E-BP1. A mechanism for rapamycin inhibition *in vivo* is proposed, in which rapamycin inhibits mTOR's ability to activate downstream effectors—but not its intrinsic catalytic activity—by blocking PA binding to the FRB domain. A tentative model for the mTOR signaling circuitry has emerged to serve as a working hypothesis (Fig. 2). The implication of PLD in mTOR signaling brings to light several candidate regulators that potentially mediate a diverse range of mTOR regulation. Awaiting future investigation is the involvement of cPKC, ARF, Rho, calcium ion, and other regulators of PA-production in mTOR signaling, in a cell type-specific and signal-specific context. In addition, potential nuclear functions of mTOR, such as DNA damage response and regulation of transcription/transcription factors, are distinct possibilities that warrant future research efforts. Moreover, the dynamics of mTOR subcellular localization remain an intriguing puzzle. Unraveling the *in vivo* correlation between mTOR membrane lo-



**Fig. 2** The new mTOR signaling network—a working hypothesis

calization, PA binding, and nuclear translocation should prove instrumental in our understanding of how this master regulator coordinates the multitude of cellular functions it governs.

*Acknowledgment.* I would like to thank Dr. Michael Coon and members of the Chen lab for insightful discussions. Funding for the work is provided by the National Institute of Health.

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# Raptor and mTOR: Subunits of a Nutrient-Sensitive Complex

D. H. Kim · D. M. Sabatini

Whitehead Institute for Biomedical Research, Nine Cambridge Center,  
Cambridge, MA 02142, USA  
*E-mail: sabatini@wi.mit.edu*

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**Abstract** mTOR/RAFT1/FRAP is the target of the FKBP12-rapamycin complex as well as a central component of a nutrient- and hormone-sensitive pathway that controls cellular growth. Recent work reveals that mTOR interacts with a novel evolutionarily conserved protein that we named raptor, for “regulatory associated protein of mTOR.” Raptor has several roles in the mTOR pathway. It is necessary for nutrient-mediated activation of the downstream effector S6K1 and increases in cell size. In addition, under conditions that repress the mTOR pathway, the association of raptor with mTOR is strengthened, leading to a decrease in mTOR kinase activity. Raptor is a critical component of the mTOR pathway that regulates cell growth in response to nutrient levels by associating with mTOR.



## 1

**Introduction**

In mammalian cells, the mTOR signaling pathway plays a major role in regulating nutrient- and hormone-sensitive cell growth (reviewed in Schmelzle and Hall 2000, Gingras et al. 2001). This pathway coordinates the synthesis of ribosomal proteins with nutrient levels and controls cap-dependent mRNA translation and has recently been shown to regulate the expression of metabolism-related genes (Peng et al. 2002). mTOR, also known as RAFT1 or FRAP, phosphorylates two regulators of mRNA translation: S6K1 and an inhibitor of translation initiation, the eIF-4E binding protein 1 (4E-BP1; Brunn et al. 1997; Burnett et al. 1998; Hara et al. 1997; Isotani et al. 1999). The activity of these mTOR effectors is regulated by nutrients, with amino-acid deprivation causing a rapid dephosphorylation of S6K1 and 4E-BP1 and decreased rates of protein synthesis (Fox et al. 1998; Hara et al. 1998). In many mammalian cell types the mTOR pathway is particularly sensitive to changes in the levels of the branched-chain amino acid leucine (Hara et al. 1998; Lynch et al. 2000). Several lines of evidence indicate that leucine-induced activation of the pathway requires a precise structural feature of the amino acid and suggest that tRNA synthetases and amino acid transporters are not upstream sensors in the mTOR pathway (Lynch et al. 2000). The exact nature of the nutrient-derived signals that regulate the mTOR pathway are unknown, but the sensitivity of the S6K1 phosphorylation state to several metabolic inhibitors suggests that mitochondrial metabolism of leucine is required for signaling by the mTOR pathway (Xu et al. 2001; Dennis et al. 2001; D.D. Sarbassov, D.-H.Kim and D.M.Sabatini, unpublished data). The recent discovery of raptor, a protein whose interaction with mTOR is sensitive to nutrients and mitochondrial function, provides insight into the molecular mechanisms with which mTOR signals to the cell growth machinery (Kim et al. 2002).

## 2

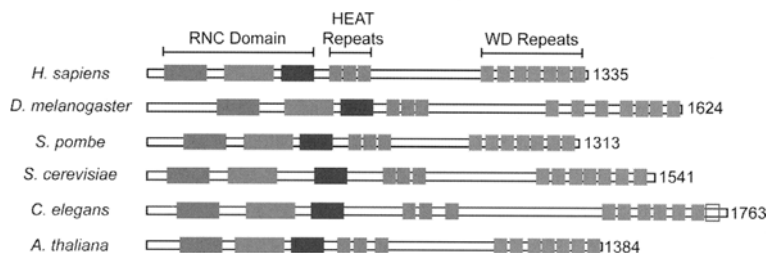
**Raptor: An Interacting Protein of mTOR**

## 2.1

**Raptor and mTOR Form a Stoichiometric Complex**

Despite extensive efforts, conventional biochemical attempts have not been successful in identifying mTOR-interacting proteins, suggesting that the interaction between mTOR and other proteins might be unstable under commonly used cell lysis solutions. Raptor was first identified in HEK293T cells by stabilizing its association with mTOR with the use of a reversible chemical cross-linker (Kim et al. 2002). Raptor was also independently identified by Hara et al. as an mTOR interacting protein (Hara et al. 2002). In the cross-linked complex, raptor is present in a stoichiometric ratio with mTOR. In the absence of a cross-linker, the interaction of raptor with mTOR is sensitive to Triton X-100 but stable in buffers containing 0.3% CHAPS, conditions with which near stoichiometric amounts of the two proteins can be recovered. Interestingly, most of the *in vitro* studies on mTOR function have been carried out on mTOR isolated from cells lysed with buffers containing Triton X-100 or NP-40, detergents that disrupt the complex even at the lowest concentrations tested. Mutagenesis studies reveal that mTOR and raptor likely make extensive contacts with each other. A stable interaction requires the entire N-terminal half of mTOR that contains the HEAT repeats and multiple regions of raptor. It is also possible to detect a weak interaction between raptor and the C-terminal half of mTOR.

The mTOR-raptor complex exists in all human cell lines tested, including the uterine cancer HeLa, B-cell lymphoma BJAB, neuroblastoma SK-N-MC, and lung cancer A549-derived cell lines, as well as in mouse primary cells and in the C2C12 and NIH3T3 cell lines (D.-H. Kim and D.M. Sabatini, unpublished data). Interestingly, northern blot analyses show that raptor is expressed in all human tissues in a pattern of abundance similar to that of mTOR, with the greatest levels of both mRNAs found in skeletal muscle, brain, kidney, and placenta. This finding suggests that both proteins may be coordinately expressed within cells. Consistent with this possibility, a reduction in the expression of either raptor or mTOR also leads to a decrease in the levels of the other protein. This mutual dependence on each other for expression was also observed between ATR, a protein which like mTOR is a member of the PIK family of kinases, and ATRIP (ATR-Interacting Protein; Cortez et al. 2001).



**Fig. 1** Raptor is an evolutionarily conserved protein in eukaryotic species. The seventh WD40 repeat of raptor in *C. elegans* is denoted with an *empty gray box* because it does not have the prototypical Trp/Asp pattern. The accession numbers for the raptor orthologues are: *D. melanogaster* (AAF46122), *S. pombe* (P87141), *S. cerevisiae* (P38873), *C. elegans* (T19183), and *A. thaliana* (NP\_187497)

## 2.2

### Raptor Is an Evolutionarily Conserved Protein

Raptor is highly conserved amongst all eukaryotes for which sequence information is available, including *D. melanogaster*, *S. pombe*, *S. cerevisiae*, *C. elegans*, and *A. thaliana* (Fig. 1). In particular, the RNC (for raptor N-terminal conserved) domain, which consists of three sequence blocks, exhibits high sequence similarity amongst raptor orthologues. Database searches reveal that the RNC domain is found only in raptor orthologues and has a high propensity to form  $\alpha$ -helices. This domain likely plays a crucial role in the mTOR pathway because mutations throughout the RNC domain disrupt the raptor–mTOR interaction. Not excluding the possibility that this domain may have enzymatic activity, its high propensity for  $\alpha$ -helicity supports the idea that it might be a structural motif that recognizes a cellular component common to all eukaryotes or might participate in mediating the interaction of raptor with mTOR. Following the RNC domain, all raptor orthologues have three HEAT repeats in the middle region of the protein and seven WD40 repeats in the C-terminal half. Both HEAT and WD40 repeats are protein–protein interaction motifs present in many eukaryotic regulatory proteins (Kobe et al. 1999; Smith et al. 1999). The WD40 domain is found in proteins involved in diverse biological processes including signal transduction, RNA processing, gene expression, vesicular trafficking, cytoskeletal assembly, and cell cycle regulation (Neer et al. 1994). Like with the RNC domain, mutagenesis studies reveal that the WD40 repeats are important for

maintaining the interaction with mTOR, but it is not yet clear whether the mutations affect the mTOR binding site on raptor or induce a conformational change not favorable to the mTOR–raptor interaction. Interestingly the raptor orthologue in fission yeast, Mip1p, interacts with Mei2p and Ste11p, key factors in meiosis and conjugation (Shinozaki-Yabana et al. 2000), perhaps indicating that in mammalian cells the raptor–mTOR complex contains additional still unidentified components.

### 3

## **Raptor Functions in mTOR-Mediated Processes**

### 3.1

#### **Raptor and Nutrient-Regulated Signaling**

The function of raptor in the mTOR signaling pathway was assessed through the use of siRNAs that specifically reduce its expression level (Kim et al. 2002). In HEK293T cells, a decrease in raptor expression attenuates leucine-stimulated phosphorylation of S6K1 to a similar degree as a reduction in mTOR achieved with an siRNA targeting mTOR. This finding suggests that raptor has a positive function in mediating the nutrient-sensitive phosphorylation of the S6K1, a process in which mTOR plays an essential role (Brown et al. 1995; Chung et al. 1992; Kuo et al. 1992). However, it seems unlikely that raptor is necessary for maintaining mTOR kinase activity because, *in vitro*, raptor-depleted mTOR exhibits robust kinase activity towards S6K1. Furthermore, previous studies using truncation and point mutants of mTOR reveal that mTOR kinase activity is necessary but not sufficient for signaling to S6K1 *in vivo* (Brown et al. 1995; Sabatini et al. 1999). Thus, the interaction of raptor with mTOR might have a positive role in mTOR function other than in maintaining an active kinase domain. A simple possibility is that raptor plays a role in stabilizing or folding mTOR, as suggested by the apparent requirement of raptor for normal mTOR expression levels. Alternatively, raptor may be involved in determining the subcellular localization of mTOR or in bringing substrates to its kinase domain.

### 3.2

#### Raptor and Cell Size Control

During the past several years substantial progress has been made in understanding the signaling mechanisms that regulate cell growth (for review, see Kozma and Thomas 2002). Genetic studies in *Drosophila* indicate that the insulin-receptor signaling pathway plays a major role in the control of growth, and loss of function alleles in components of this pathway perturb the sizes of both cells and organs (reviewed by Stocker and Hafen 2000). It is still controversial as to whether TOR is a component of insulin receptor signaling pathway, but, in mammalian cells, S6K1 is clearly a common downstream effector of both the mTOR and insulin-receptor signaling pathways. Thus, S6K1 likely plays a fundamental, evolutionarily conserved role in cell growth regulation, as suggested by the finding that mice and flies lacking S6K1 have smaller bodies with smaller cells (Montagne et al. 1999; Shima et al. 1998). In tissue culture cells, inhibition of the mTOR pathway with rapamycin reduces the size of many mammalian cells in all phases of the cell cycle (Kim et al. 2002; S.M. Ali and D.M. Sabatini, unpublished data).

A positive role for raptor and mTOR in the control of mammalian cell size can also be demonstrated using siRNAs targeting the raptor and mTOR mRNAs (Kim et al. 2002). In actively dividing HEK293T cells, reductions in the levels of raptor or mTOR shrink cell size to similar extents. Furthermore, reductions in the expression of either protein also significantly diminish the increase in size that occurs when cells emerge from prolonged nutrient deprivation or confluence-induced growth arrest. The reductions in raptor levels that reduce cell size are always accompanied by a reduction in the phosphorylation of S6K1 at threonine 389, a residue phosphorylated by mTOR in vitro (Burnett et al. 1998; Isotani et al. 1999).

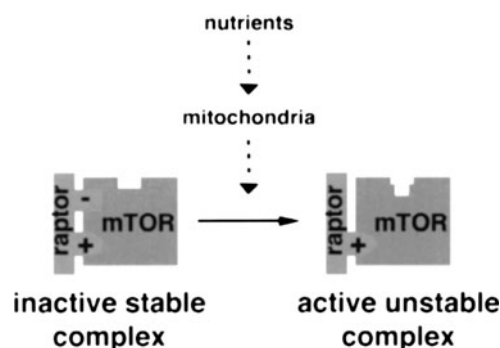
## 4

### Regulation of the Raptor–mTOR Interaction

#### 4.1

##### Nutrients

Cellular nutrient levels and metabolic conditions regulate the stability of the mTOR–raptor interaction (Kim et al. 2002). Compared to nutrient-



**Fig. 2** Model for the mTOR–raptor regulation by upstream signals as explained in the text

rich media, greater amounts of raptor are bound to immunopurified mTOR when the complex is isolated from cells incubated for short periods of time in media without amino acids or glucose. Furthermore, the increased amount of raptor bound to mTOR correlates with a lower mTOR kinase activity *in vitro* towards S6K1 and 4E-BP1 substrates. This inverse correlation between the amount of raptor bound to mTOR and the *in vitro* mTOR kinase activity suggests a negative function for raptor in regulating mTOR kinase activity. Supporting this relationship, overexpression of wild-type raptor in HEK293T cells increases the amount of raptor bound to mTOR and decreases the *in vitro* mTOR kinase activity, effects not seen with overexpression of a mutant raptor that cannot bind to mTOR. In addition, the overexpression of wild-type raptor decreases the *in vivo* phosphorylation state of S6K1 and increases the amount of 4E-BP1 bound to eIF-4E. These results provide *in vivo* correlates of the inhibitory effects of raptor on the *in vitro* mTOR kinase activity.

Despite the evidence for a negative role for raptor in modulating the mTOR kinase activity, the siRNA experiments suggest that *in vivo* raptor also has a positive role in the mTOR pathway because reductions in raptor levels decrease S6K1 phosphorylation, cell size, cell growth, and mTOR expression. A model in which the raptor–mTOR complex exists in two nutrient-determined states can account for the proposed dual functions of raptor in the mTOR pathway (Fig. 2). This model proposes that within cells raptor and mTOR associate under all conditions, but that the stability of the complex during *in vitro* isolation varies depending on nutrient availability and mitochondrial function. In this model,

raptor and mTOR are held together by two interactions: a “constitutive” and a “nutrient-sensitive” interaction. The constitutive interaction has a positive role, is not sensitive to nutrient conditions, and maintains the two proteins together irrespective of nutrient levels. In the absence of nutrients, the nutrient-sensitive interaction forms (or is strengthened), leading to a stabilization of the complex so that under nutrient-poor conditions high amounts of raptor are recovered bound to mTOR. On the other hand, under nutrient-rich conditions, the nutrient-sensitive interaction is weak so that most of the complex falls apart during *in vitro* isolation. Several experimental findings support the proposed model. When mTOR is isolated from cells lysed in the presence of a cross-linker, stoichiometric amounts of raptor are always bound to mTOR, irrespective of nutrient conditions. Furthermore, two raptor mutants were found that selectively perturb the nutrient-sensitive interaction. The model proposes that the nutrient-sensitive mTOR–raptor interaction negatively regulates the catalytic activity of mTOR. The molecular mechanism for this regulation is not known, but may include inducing a conformational change in the mTOR kinase domain, sterically preventing substrates from accessing the mTOR active site or affecting the function of another still unidentified mTOR interaction protein(s). In addition, the model proposes that a nutrient- and mitochondrially derived signal regulates the nutrient-sensitive interaction between raptor and mTOR.

#### 4.2

##### Rapamycin

Rapamycin is an immunosuppressive drug used to block the acute rejection of transplanted organs (Saunders et al. 2001) and is in clinical trials as a potential anticancer agent (Dudkin et al. 2001; Hidalgo and Rowinsky 2000). The sensitivity of a biological process to rapamycin is also routinely used as a test of whether the mTOR pathway participates in that process. Rapamycin is known to act through mTOR and was instrumental in its original identification (Brown et al. 1994; Chiu et al. 1994; Kunz et al. 1993; Sabatini et al. 1994; Sabers et al. 1995), but exactly how the drug exerts its inhibitory effects on the mTOR pathway remains poorly understood. The recent finding that rapamycin potently destabilizes the mTOR–raptor interaction (Kim et al. 2002) may shed light on its molecular mechanism of action. The drug disrupts the mTOR–raptor association either when added to live cells or *in vitro* after cell lysis, sug-

gesting that it acts by sterically perturbing the interaction rather than by blocking the formation of a posttranslation modification needed for the interaction. However, from cross-linking experiments, it is clear that rapamycin does not dissociate mTOR and raptor within the cell, but rather weakens the interaction so that it is unstable after cell lysis. How the rapamycin-FKBP12 complex destabilizes the interaction is not clear, particularly as the binding sites on mTOR for raptor and rapamycin-FKBP12 are far apart. Although nutrient deprivation and rapamycin both inhibit the mTOR pathway, they have opposite effects on the stability of the mTOR-raptor interaction, suggesting that further complexity exists in the mTOR signaling mechanism. Moreover, because nutrient levels change the EC<sub>50</sub> for rapamycin-induced disruption of the mTOR-raptor complex, it is likely that cross talk exists between rapamycin and nutrients. In addition, recent findings suggest that FKBP12-rapamycin also affects the interaction of phosphatidic acid with mTOR (Fang et al. 2001), although how it affects the raptor-mTOR complex is unknown.

#### 4.3

##### Upstream Signals That Regulate the Raptor-mTOR Interaction

The sensitivity of the raptor-mTOR interaction to nutrients and mitochondrial function provides a potential mechanism for how the mTOR pathway responds to changes in nutrient conditions. An unanswered and critical question is whether the raptor-mTOR complex responds to one or several distinct nutrient-derived signals. Current findings can be interpreted in either manner. The similar effects of leucine and glucose levels on the raptor-mTOR interaction, the mTOR activity, and the activity of the pathway may indicate that the complex responds to multiple signals. On the other hand, signals derived from different nutrients might converge on an element upstream of mTOR to generate a common signal that affects the mTOR-raptor complex.

Even though the exact nature of upstream signals is not known, the metabolism of nutrients by the mitochondria is likely to be involved. It has been well documented that the mitochondrial metabolism of nutrients is necessary to activate the mTOR pathway (Dennis et al. 2001; Xu et al. 2001). For example, disruption of mitochondrial function using different small molecules like antimycin A, a mitochondrial uncoupler (Bernard and Cockrell 1979), or valinomycin, an electron transport inhibitor (Wolvetang et al. 1994), leads to a strengthening of the raptor-



mTOR interaction and inhibition of the mTOR pathway (Kim et al. 2002). Several reagents that specifically inhibit the metabolism of amino acids or glucose by the mitochondria also regulate the interaction as well as the mTOR pathway (D.-H. Kim, D.D. Sarbassov and D.M. Sabatini, unpublished data).

Recently, Dennis et al. reported that mTOR may be a homeostatic sensor of intracellular ATP levels (Dennis et al. 2001). They suggest that the decrease in intracellular ATP concentration caused by 2-deoxyglucose, an inhibitor of the glycolysis, also decreases mTOR kinase activity. 2-Deoxyglucose also affects the raptor-mTOR interaction as does nutrient deprivation or mitochondrial inhibitors (Kim et al. 2002). Thus, ATP is a candidate upstream signaling molecule affecting the mTOR-raptor interaction. This would be consistent with the stabilizing effects of antimycin A and valinomycin on the raptor-mTOR interaction because they both perturb mitochondrial function and decrease ATP levels. As changes in amino acid levels do not affect gross cellular levels of ATP (Dennis et al. 2001), it has been proposed that mTOR must sense more than one signal. This is consistent with the diverse repertoire of signals known to affect the pathway, including conditions such as oxidative stress caused by  $H_2O_2$  treatment, which stabilizes the mTOR-raptor interaction and inhibits the mTOR kinase. It is also interesting that the mTOR-raptor complex is sensitive to the detergents used to lyse cells, being destabilized by low concentrations of Triton X-100 and NP-40, but not CHAPS or Tween-20. This may indicate that a hydrophobic molecule or a hydrophobic interface(s) on the two proteins plays a role in maintaining the interaction.

In addition to nutrients, insulin also regulates downstream components of the mTOR pathway, like S6K1 (Lawrence and Brunn 2001), and mTOR is a substrate for the insulin-regulated kinase PKB/Akt (Scott et al. 1998). Treatment of cells with insulin, however, seems not to affect the raptor-mTOR interaction and the complex is still regulated by nutrients even in the absence of serum (D.-H. Kim, D.M. Sabatini, unpublished data). Perhaps insulin affects properties of raptor that are still not studied, such as its phosphorylation state, and has subtler effects on the raptor-mTOR interaction than nutrients. On the other hand, insulin may signal to S6K1 through mechanisms that do not involve raptor, such as mTOR phosphorylation (Scott et al. 1998; Sekulic et al. 2000) or phosphatidic acid levels (Fang et al. 2001).

## 5

**Concluding Remarks**

The discovery of raptor and its nutrient-regulated interaction with mTOR provides a potential mechanism for control of the mTOR pathway by nutrients and mitochondrial function. An interesting observation is that raptor appears to have both positive and negative functions in the mTOR pathway. Raptor has a positive role in mTOR-mediated processes, such as S6K1 control and cell size regulation, but the stable association of the two proteins induced by nutrient-poor conditions also leads to an inhibition of mTOR kinase activity. The sensitivity of the raptor-mTOR interaction to diverse conditions, such as amino acid and glucose levels, oxidative stress, and mitochondrial metabolism suggests an integral role for the raptor-mTOR complex in signaling nutrient and environmental conditions to downstream effectors. The exact nature of the upstream signals that regulate the interaction and the mechanism by which it influences mTOR activity remain to be elucidated. In addition, how the mTOR pathway integrates nutrient- and growth factor-derived signals continues to be an unanswered question.

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# Kinase Activities Associated with mTOR

K. Yonezawa · K.-I. Yoshino · C. Tokunaga · K. Hara

Biosignal Research Center, Kobe University, 657-8501, Kobe, Japan

E-mail: yonezawa@kobe-u.ac.jp

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**Abstract** Although mTOR is a member of the PI-kinase-related kinase family, mTOR possesses serine-threonine protein kinase activities, which phosphorylate itself and exogenous substrates. mTOR autophosphorylates in vitro and is phosphorylated in vivo on serine residues. Ser<sup>2481</sup>, which is located in a His-Ser-Phe motif near the conserved carboxyl-terminal mTOR tail, has been reported as an autophosphorylation site in vivo and in vitro. The significance of the autophosphorylation remains unclear. Another phosphorylation site on mTOR in vivo is Ser<sup>2448</sup>. This site appears not to be an autophosphorylation site but a site potentially phosphorylated by protein kinase B (PKB). mTOR immunopurified from culture cells or tissues phosphorylates in vitro p70 S6 kinase (p70)  $\alpha$  and p70 $\beta$ , mainly on Thr<sup>412</sup> or Thr<sup>401</sup>, respectively, located in a Phe-Thr-Tyr motif. Another exogenous substrate phosphorylated by immunopurified mTOR in vitro is eIF4E-binding protein 1 (4E-BP1) at sites corresponding to those phosphorylated in vivo during insulin stimulation in a Ser/Thr-Pro motif. Recently, raptor, a 150-kDa TOR-binding protein that contains a carboxyl-terminal WD-repeat domain, was discovered as a scaffold for the mTOR-catalyzed phosphorylation of 4E-

BP1 and for the mTOR-mediated phosphorylation and activation of p70 $\alpha$ . Other potential substrates phosphorylated by mTOR are nPKC $\delta$ , nPKC $\epsilon$ , STAT3, and p53. The requirement of raptor for binding to and phosphorylation by mTOR of these potential substrates would clarify their physiological importance in the mTOR signaling pathway.

## 1

### Introduction

The macrolide immunosuppressant rapamycin is known to cause dephosphorylation and inactivation of translational effectors, such as p70 S6 kinase (p70) and eIF4E-binding protein 1 (4E-BP1; Azpiazu et al. 1996; Beretta et al. 1996; Chung et al. 1992; Kuo et al. 1992; Price et al. 1992). Rapamycin, in complex with the cytosolic FK506-binding protein (FKBP12), binds to the mammalian target of rapamycin mTOR (also known as FRAP, RAFT1, or RAPT; Brown et al. 1994; Chiu et al. 1994; Sabatini et al. 1994; Sabers et al. 1995). The protein mTOR contains a kinase domain near its C terminus, most closely related to the PI-kinase-related kinase family, which includes ATM, ATR, and DNA-PK (Keith and Schreiber, 1995; Takahashi et al. 2000; Zakian, 1995). The other characteristic structure includes N terminal HEAT repeats, FAT/FATC domains surrounding the kinase domain, and the FKBP-rapamycin binding (FKB) domain. Evidence supporting the mTOR kinase as the rapamycin-sensitive element responsible for the inhibition of translational effectors is the finding that a mutant of mTOR (ST-mTOR) that no longer binds the FKBP12/rapamycin complex, protects coexpressed p70 or 4E-BP1 from rapamycin-induced dephosphorylation and/or inhibition, whereas the kinase-dead mutant of ST-mTOR (ST/NK-mTOR) does not (Brown et al. 1995; Brunn et al. 1997b; Hara et al. 1997; Minami et al. 2001). Although mTOR is a member of PI-kinase-related kinase family, it possesses serine-threonine protein kinase activities, which phosphorylate itself and exogenous substrates. In this chapter, the kinase activities associated with mTOR will be discussed.

## 2

### Autophosphorylation of mTOR

mTOR autophosphorylates in vitro and is phosphorylated in vivo on serine residues (Brown et al. 1995; Peterson et al. 2000). To date, a single

autophosphorylation site has been reported (Peterson et al. 2000). This site, Ser<sup>2481</sup>, is located in a His-Ser-Phe motif near the conserved carboxyl terminal mTOR tail. Phosphorylation of wild-type but not kinase-inactive mTOR occurs at Ser<sup>2481</sup> in vivo, suggesting that Ser<sup>2481</sup> phosphorylation is a marker of mTOR autokinase activity in cells. Although the autophosphorylation of this site occurs in vivo and in vitro, its significance remains unclear. The mutation of Ser<sup>2481</sup> to Ala does not affect the ability of ST-mTOR to protect p70 $\alpha$  from rapamycin-induced dephosphorylation. The autophosphorylation of Ser<sup>2481</sup> is inhibited by wortmannin (100 nM) but not by rapamycin treatment or amino acid deprivation, treatments that lead to acute dephosphorylation of 4E-BP1 and p70 $\alpha$ . These results suggest that mTOR-responsive dephosphorylation of 4E-BP1 and p70 $\alpha$  occurs through not only inhibition of intrinsic mTOR kinase activity but also another mechanism.

Another phosphorylation site on mTOR in vivo is Ser<sup>2448</sup>. This site appears not to be an autophosphorylation site but a site potentially phosphorylated by protein kinase B (PKB). Insulin induces phosphorylation on Ser<sup>2448</sup> and this effect is blocked by wortmannin but not rapamycin. Amino acid starvation rapidly attenuated the reactivity of the Ser<sup>2448</sup> phosphospecific antibodies with mTOR and this could not be restored by either insulin stimulation of cells or incubation with PKB in vitro (Nave et al. 1999).

### 3

#### The Kinase Activity Toward p70 Associated with mTOR

At least two genes encode p70; the p70 gene product first identified was called p70 $\alpha$  (Banerjee et al. 1990; Kozma et al. 1990) and the more recently described gene product has been designated p70 $\beta$  (Gout et al. 1998; Koh et al. 1999; Lee-Fruman et al. 1999; Shima et al. 1998). Overall sequence and structure of p70 $\beta$  are very close to those of p70 $\alpha$ 1, with 70% identity and 85% similarity at the protein level. The p70 $\alpha$  and p70 $\beta$  genes are expressed as two polypeptides designated  $\alpha$ 1 and  $\alpha$ 2, and  $\beta$ 1 and  $\beta$ 2, respectively;  $\alpha$ 1 is 525 amino acids in length and is identical to  $\alpha$ 2 starting at  $\alpha$ 1 amino acid 24;  $\beta$ 1 is 495 amino acids in length and is identical to  $\beta$ 2 starting at  $\beta$ 1 amino acids 14.

In response to insulin or mitogens, the p70 $\alpha$  activity dramatically increases through the multisite phosphorylation in vivo (Avruch et al. 2001). In addition to regulation by insulin or mitogens, nutrients (espe-

cially amino acids) recently have been discovered to regulate the phosphorylation and activation of p70 $\alpha$  and to be necessary for insulin or mitogen regulation of p70 $\alpha$  (Fox et al. 1998; Hara et al. 1998; Patti et al. 1998; Shigemitsu et al. 1999; Wang et al. 1998; Xu et al. 1998). The multi-site phosphorylation is directed at three separate domains as follows: a set of Ser/Thr-Pro motifs clustering in the autoinhibitory pseudosubstrate domain (Ferrari et al. 1992; Price et al. 1991); Ser<sup>394</sup> and Thr<sup>412</sup> located in the kinase extension domain (Moser et al. 1997; Pearson et al. 1995) and Thr<sup>252</sup> in the activation loop of the catalytic domain (Weng et al. 1995). Immunopurified from culture cells or tissues, mTOR phosphorylates p70 $\alpha$  (mainly on Thr<sup>412</sup> located in a Phe-Thr-Tyr motif) purified from *E. coli* and from mammalian cells in vitro (Burnett et al. 1998; Isotani et al. 1999). A portion of mTOR-catalyzed p70 $\alpha$  phosphorylation is directed to the carboxyl terminal tail, at least half of which is into Thr<sup>444</sup>/Ser<sup>447</sup> located in the Ser/Thr-Pro motifs in the autoinhibitory pseudosubstrate domain (Isotani et al. 1999). In addition, p70 $\alpha$  is phosphorylated on Thr<sup>252</sup> by 3-phosphoinositide-dependent protein kinase (PDK1; Alessi et al. 1998; Pullen et al. 1998). Importantly, immunopurified mTOR activates mammalian recombinant p70 $\alpha$  in a synergistic manner with PDK1 (Isotani et al. 1999). In the case of p70 $\beta$ , possible phosphorylation sites are well conserved; Thr<sup>241</sup>, Ser<sup>383</sup> and Thr<sup>401</sup> in p70 $\beta$ 1 correspond to Thr<sup>252</sup>, Ser<sup>394</sup> and Thr<sup>412</sup> in p70 $\alpha$ 1, respectively. Thr<sup>401</sup> located in a Phe-Thr-Tyr motif appears to be a main mTOR-catalyzed phosphorylation site. The kinase activity of p70 $\beta$  was less sensitive to the inhibition induced by rapamycin, wortmannin, and amino acid withdrawal than was that of p70 $\alpha$  (Gout et al. 1998; Minami et al. 2001). Mutational analysis revealed that the phosphorylation of Thr<sup>241</sup> and Thr<sup>401</sup> in p70 $\beta$ 1 was indispensable for the kinase activity. However, in contrast to p70 $\alpha$ , a p70 $\beta$ 1 mutant in which Ser<sup>383</sup> was substituted with Gly (Ser383Gly) still retained nearly half the maximal activity. Sequential phosphorylation of mammalian recombinant wild-type and Ser383Gly mutant of p70 $\beta$ 1 with immunopurified mTOR and PDK1 in vitro on Thr<sup>401</sup> and Thr<sup>241</sup>, respectively, synergistically activated their kinase activities similar to p70 $\alpha$ .

In the case of p70 $\alpha$ , mTOR alone gives some activation. Phosphorylation of the Ser/Thr-Pro sites within the carboxyl-terminal 104 amino acids may improve access of PDK1. In addition, by phosphorylating Thr<sup>412</sup>, mTOR strongly promotes the ability of PDK1 to phosphorylate

Thr<sup>252</sup>. Phosphorylation of mTOR acts primarily in a “priming” role rather than as a sole activator.

#### 4

#### The Kinase Activity Toward 4E-BP1 Associated with mTOR

Another exogenous substrate phosphorylated by immunopurified mTOR is 4E-BP1. The latter binds to the eIF-4E (7-methyl-guanosine mRNA cap-binding protein) and prevents eIF4E from binding to a scaffold protein eIF-4G, thereby inhibiting the formation of the active translational complex, eIF-4F (Sonenberg, 1996). Insulin, mitogens, or nutrients, especially amino acids, stimulate the phosphorylation of 4E-BP1, resulting in the dissociation and disinhibition of eIF-4E.

Either mTOR or an mTOR-associated kinase has been shown to phosphorylate a set of five Ser/Thr-Pro motifs of human 4E-BP1—Thr<sup>37</sup>, Thr<sup>46</sup>, Ser<sup>65</sup>, Thr<sup>70</sup>, and Ser<sup>83</sup>—directly *in vitro* at sites corresponding to those phosphorylated *in vivo* during insulin stimulation (Brunn et al. 1997a; Fadden et al. 1997). Gingras et al. have shown that phosphorylation of Thr<sup>37</sup> and Thr<sup>46</sup> serve as a priming event, allowing for the succeeding serum-induced phosphorylation of the remaining 4E-BP1 sites (Gingras et al. 1999). This priming step is followed by phosphorylation of Thr<sup>70</sup> and, finally, of Ser<sup>65</sup> (Gingras et al. 2001).

The mechanism by which mTOR phosphorylates 4E-BP1 appears to be different from that by which it phosphorylates p70 $\alpha$  and p70 $\beta$  *in vitro*. First, mTOR-catalyzed 4E-BP1 phosphorylation *in vitro* is severely reduced if the mTOR immunoprecipitate is washed with detergents such as NP-40 or CHAPS, whereas mTOR-catalyzed p70 phosphorylation is modestly reduced by the same washing (Isotani et al. 1999; Nishiuma et al. 1998). Second, immunopurified mTOR catalyzes phosphorylation exclusively on the Ser/Thr-Pro motif in 4E-BP1 (Gingras et al. 1999; Mothe-Satney et al. 2000), whereas a Phe-Thr-Tyr motif is the major site of mTOR-catalyzed phosphorylation on p70 $\alpha$  and p70 $\beta$  (Burnett et al. 1998; Isotani et al. 1999; Minami et al. 2001). Although this remarkably broad specificity suggests the operation of more than one kinase, all these mTOR-catalyzed phosphorylations are abolished by direct addition *in vitro* of a rapamycin/FKBP complex.



## 5

**Discovery of Raptor, a Binding Partner of mTOR**

We then explored the possibility that the mTOR-catalyzed phosphorylation of 4E-BP1 *in vitro* requires the participation of additional, detergent-sensitive molecules that are copurified in mTOR immunoprecipitate.

Using biochemical purification steps, we have discovered raptor, a 150-kDa TOR-binding protein that contains a carboxyl-terminal WD-repeat domain (Hara et al. 2002). Kim et al. (2002) have also identified raptor using the chemical crosslinker. Raptor forms detergent-sensitive complexes with mTOR and also binds 4E-BP1 and p70 $\alpha$  in a detergent-insensitive manner. The binding of raptor to mTOR is absolutely necessary for the mTOR-catalyzed phosphorylation of 4E-BP1 *in vitro*, and strongly enhances the TOR kinase activity toward p70 $\alpha$ . The raptor polypeptide copurifies in part from cell extracts with 4EBP1 on 7-methyl-GTP sepharose. Treatment of cells with rapamycin or amino acid withdrawal increases the recovery of 4E-BP1 and raptor on 7-methyl-GTP sepharose, whereas insulin strongly inhibits the recovery of both 4E-BP1 and raptor. Overexpression of wild-type raptor or a mutant lacking the WD-repeat domain each inhibits the phosphorylation of coexpressed 4E-BP1 and the activation of p70 $\alpha$ . RNA interference (RNAi) of raptor results in the reduction of both expression of raptor *in vivo* and mTOR-catalyzed 4E-BP1 phosphorylation *in vitro*. Thus, raptor functions as an essential scaffold for the mTOR-catalyzed phosphorylation of 4E-BP1 and the mTOR-mediated phosphorylation and activation of p70 $\alpha$  (Hara et al. 2002).

## 6

**Potential Substrates Phosphorylated by mTOR**

The AGC (A, G, and C) kinase subfamily also includes p70. The kinase extension domain of p70 $\alpha$  is 40% identical to homologous segments in the PKCs, PKBs, and Rsk (N-terminal catalytic domain) subgroup of AGC kinases (Avruch et al. 2001). The hydrophobic sites in the kinase extension domain is also conserved in AGC kinases. Phosphorylation at the hydrophobic site of nPKC $\delta$ , Ser<sup>662</sup>, in the kinase extension domain is controlled by a pathway-involved mTOR, because the rapamycin-induced block of its phosphorylation is overcome by co-expression of

ST-mTOR (Parekh et al. 1999). Consistent with this role of mTOR, amino acid deprivation selectively inhibits the serum-induced phosphorylation of the Ser<sup>662</sup> in nPKC $\delta$ . It is also established that nPKC $\epsilon$  behaves in a manner similar to nPKC $\delta$  with respect to phosphorylation at its hydrophobic site, Ser<sup>719</sup>, in the kinase extension domain (Parekh et al. 1999).

The second potential substrate for mTOR is signal transducer and activator of transcription 3, STAT3. In ciliary neurotrophic factor (CNTF)-stimulated neuroblastoma cells, mTOR appears to be responsible for phosphorylation of Ser<sup>727</sup> in the Ser/Pro motif of STAT3 (Yokogami et al. 2000). Rapamycin treatment of cells transfected with a STAT-responsive promoter reporter decreased activation of the reporter to the same degree as a STAT3 Ser727Ala mutant. The ability of mTOR to contribute to activation of STAT3 extends the function of mTOR in mammalian cells to include transcriptional regulation.

The third potential substrate for mTOR is p53. Castedo et al. delineated the apoptotic pathway resulting from human immunodeficiency virus (HIV)-1 envelope glycoprotein (Env)-induced syncytia formation in vitro and in vivo (Castedo et al. 2001). Immunohistochemical analysis demonstrated the presence of phosphorylated Ser<sup>15</sup> of p53 as well as the preapoptotic marker tissue transglutaminase in syncytium in the apical light zone (T-cell area) of lymph nodes, as well as in peripheral blood mononuclear cells, from HIV-1-positive but not HIV-1-negative donors. The presence of these markers correlated with viral load (HIV-1 RNA levels). Quantitative immunoblot analysis showed that phosphorylation of Ser<sup>15</sup> in the Ser-Gln motif of p53 in response to HIV-1 Env is mediated by mTOR and not by other phosphatidylinositol kinase-related kinases, and it is accompanied by downregulation of protein phosphatase 2A. The phosphorylation is significantly inhibited by rapamycin. Immunofluorescence microscopy indicated that mTOR is enriched in syncytial nuclei and that the nuclear accumulation precedes the phosphorylation of Ser<sup>15</sup> of p53. Thus the authors concluded that HIV-1 Env-induced syncytium formation leads to apoptosis via a pathway that involves phosphorylation of Ser<sup>15</sup> of p53 by mTOR, followed by activation of BAX, mitochondrial membrane permeabilization, release of cytochrome C, and caspase activation.

**Table 1** Reported substrates for mTOR kinase

Substrates	Phosphorylation sites	Motif	Detergent sensitivity in vitro
mTOR	Ser2481	His-Ser-Phe	?
p70 $\alpha$ 1	Thr412	Phe-Thr-Tyr	modestly sensitive
Thr444/Ser447	Ser/Thr-Pro	?	
p70 $\beta$ 1	Thr401	Phe-Thr-Tyr	?
4E-BP1	Thr37/Thr46/Ser65/Thr70/ Ser83	Ser/Thr-Pro	Sensitive
PKC $\delta$	Ser662	Phe-Ser-Phe	?
PKC $\epsilon$	Ser719	Phe-Ser-Tyr	?
STAT3	Ser727	Ser-Pro	?
p53	Ser15	Ser-Gln	?

## 7

**Future Directions**

An intriguing and unsolved aspect of mTOR function is the apparent ability of its single catalytic domain to catalyze phosphorylation of Ser/Thr-Pro sites, such as those on eIF-4E-BP1 and p70 $\alpha$  Thr<sup>444</sup>/Ser<sup>447</sup>, as well as Phe/His-Ser/Thr-Tyr/Phe sites such as mTOR Ser<sup>2481</sup>, p70 $\alpha$  Thr<sup>412</sup>, and p70 $\beta$  Thr<sup>401</sup> (Table 1). Although these two kinds of mTOR kinase activity appear differently dependent on raptor in vitro, the detergent-sensitive binding partner of mTOR, they are both inhibited by rapamycin/FKBP-12 in vitro and by mutation in the mTOR kinase domain. Assuming both activities are physiologically meaningful, such a breadth in substrate specificity is relatively unprecedented among protein kinases.

We are now exploring the possibility that the mTOR-dependent phosphorylation of 4E-BP1 and/or p70 in vitro requires the participation of additional kinases that are copurified in mTOR or raptor immunoprecipitate. In addition, investigation of autophosphorylation sites of mTOR other than Ser<sup>2481</sup> are now in progress with use of mass spectrometry analysis.

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# mTOR: A Mediator of Intracellular Homeostasis

A. Jaeschke<sup>1</sup> · P. B. Dennis<sup>2</sup> · G. Thomas<sup>3</sup>

<sup>1</sup> Howard Hughes Medical Institute and Program in Molecular Medicine,  
University of Massachusetts Medical School, Worcester, MA 01605, USA

<sup>2</sup> Wright State University, 3640 Colonel Glenn Highway, Dayton, OH 45435, USA

<sup>3</sup> Friedrich Miescher Institute, Maulbeerstrasse 66, 4058 Basel, Switzerland  
*E-mail: gthomas@fmi.ch*

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**Abstract** Earlier studies have shown that mTOR plays a key role in ribosome biogenesis. In bacteria, amino acids and ATP levels independently control ribosome biogenesis. Here, we describe recent findings demonstrating that homeostatic levels of amino acids, most notably branched-chain amino acids, and ATP, independently regulate the activity of mTOR. Unlike the effects of amino acids, the effects of ATP appear to be direct. Based on these findings we propose a model by which tumor cells existing in the anaerobic environment may have an advantage in growth by exploiting the rapid, although less efficient, production of ATP to drive growth via the mTOR signaling pathway.



## 1

**Introduction**

The survival of organisms is dependent on their ability to maintain tissue homeostasis in response to changes in their external environment. Generally, this is achieved by signaling networks that consist of sensors, signal transducers and effector molecules, which control specific intracellular responses. The phosphatidylinositol kinase-related family of protein kinases (PIKs) is emerging as being critical for sensing and responding to environmental factors that impinge on the ability of an organism to survive. The family includes ataxia telangiectasia mutated gene (ATM) and ATM related (ATR), as well as DNA-dependent protein kinase (DNAPK), which are all involved in maintaining genome integrity in response to DNA damage (Khanna and Jackson 2001). In contrast, the mammalian target of rapamycin (mTOR) resides at the interface between sensing nutrients and regulating major metabolic responses (Dennis and Thomas 2002; Gingras et al. 2001). In answer to growth factors and nutrients, mTOR positively regulates major anabolic processes such as ribosome biogenesis (Powers and Walters 1999; Mahajan 1994) and protein synthesis (Dennis and Thomas 2002; Gingras et al. 2001), while suppressing catabolic processes such as autophagy (Dennis et al. 1999; Kamada et al. 2000). As ribosome biogenesis and protein synthesis are two of the major energy consuming processes in the cell, these metabolic events must be closely monitored in response to hormonal and nutrient signals. mTOR is emerging as a key integrator of mitogen and nutrient signals, adjusting the rate of protein synthesis to the availability of translational precursors and energy state, thereby maintaining intracellular homeostasis (Dennis et al. 2001).

## 2

**Regulation of mTOR Activity**

An unsettled issue concerning mTOR signaling has been the mechanism by which its activity is regulated. mTOR is made up of a number of evolutionarily conserved subdomains, each of which may have a potential function. The carboxyl terminal third of mTOR contains a catalytic domain that bears homology with the PI3K family of lipid kinases (Dennis and Thomas 2002). However, the catalytic domain is closer to those of the other PIK family members, ATM, ATR, and DNAPK (Dennis and

Thomas 2002). A potential regulatory region is the FKBP/rapamycin-binding (FRB) domain, adjacent to the catalytic domain, in which the FKBP12/rapamycin complex binds and inhibits mTOR function. Although no endogenous ligand was found that increases FKBP12 binding to mTOR, microinjection of the FRB domain into human cells resulted in G1 arrest (Vilella-Bach et al. 1999), suggesting that the FRB domain may provide a docking site for a molecule that modulates mTOR activity. Support for this comes from the recent observation that the second messenger phosphatidic acid binds to the FRB domain and potentiates mTOR protein kinase activity (Fang et al. 2001). In addition to FRB and catalytic domains, mTOR contains a series of HEAT repeats (named after the proteins containing these repeats: Huntingtin, EF3, A subunit of PP2A, and TOR), which are thought to be involved in protein-protein interactions and may provide potential protein-docking sites (Anrade and Bork 1995; Anrade et al. 2001). It is still unclear what role the HEAT repeats play in the regulation of mTOR function. Although truncation of the HEAT repeats inhibits the ability of mTOR to signal downstream, a similar effect is observed with a truncation of the amino acids immediately preceding the HEAT repeats (Brown et al. 1995). Potentially consistent with interacting proteins required for mTOR signaling, co-expression of a kinase-inactive mTOR allele together with a wild-type mTOR reporter lowers the activity of the reporter (A. Jaeschke, P.B. Dennis, G. Thomas, unpublished data). Similarly, co-expression of the same dominant interfering allele in which the amino terminus as well has been deleted, including the HEAT repeats, also lowers the mTOR activity of the reporter (A. Jaeschke, P.B. Dennis, G. Thomas, unpublished data). These data suggest that dominant interfering alleles of mTOR are capable of titrating a signal necessary for mTOR activity. In addition, these studies have led to the suggestion that such an effector interacts with the kinase domain (see the chapter by Kim and Sabatini, this volume). In addition to the binding of effector molecules, regulation of mTOR may involve phosphorylation. It has been reported that PKB phosphorylates and modulates mTOR activity in a mitogen-dependent manner, however, mutagenesis of the identified phosphorylation sites had no effect on the ability of mTOR to signal to its effectors, S6K and 4E-BP1 (Sekulic et al. 2000). Furthermore this site is absent in the *Drosophila* orthologue of mTOR, dTOR (Oldham et al. 2000), and recent biochemical, genetic, and pharmacological studies suggest that dTOR is not a downstream effector of dPKB (Radimerski et al. 2002). Finally, a number of investigators have

argued that mitogen treatment does not cause a change in mTOR kinase activity toward either S6K or 4E-BP1 when measured *in vitro* (Dennis et al. 2001; Gingras et al. 1999), suggesting that it may instead influence the composition and signaling capabilities of factors that are bound to mTOR. Indeed, a prominent emerging facet of mTOR signaling involves the permissive regulation of mTOR function controlled by intracellular levels of amino acids and ATP (Dennis et al. 2001; Hara et al. 1998; see below).

### 3

#### **Regulation of Protein Synthesis in Prokaryotes**

In bacteria, protein synthesis and cell growth are closely linked through ribosome biogenesis, which is independently controlled by amino acid and energy availability (Roberts 1997; Gaal et al. 1997). Amino acid deprivation triggers the so-called stringent response, leading to the inhibition of rRNA and tRNA synthesis. In the stringent response, amino acid deprivation reduces the availability of charged tRNAs and causes ribosomes to stall in translation at the cognate mRNA codon. Stalling leads to the activation of RelA, a ribosome-associated protein, which produces a guanosine triphosphate (GTP) derivative, ppGpp or “magic spot,” from GTP and ATP (Roberts 1997). ppGpp binds to RNA polymerase and inhibits the production of ribosomal RNA (rRNA). In contrast, a decrease of intracellular ATP/GTP levels attenuates rRNA synthesis due to the decay of open promoter complexes (Gaal et al. 1997). The promoters for rRNA genes form unusually unstable complexes that are particularly sensitive to the concentration of the initiating nucleotide, ATP or GTP. This model implies that the rate of protein synthesis is set to the maximum allowed by the nutritional state. That is to say, RNA synthesis (and therefore the capacity for protein synthesis) will diminish only when protein synthesis has depleted cellular ATP enough to reduce the rate of transcriptional initiation at RNA promoters. On the other hand, stringent control would serve to inhibit futile RNA synthesis if protein synthesis is stalled by amino acid deprivation while energy is abundant. Thus, amino acids and energy are sensed by two distinct mechanisms, which converge on ribosome biogenesis and cell growth. Studies in yeast and metazoan systems have indicated that, like bacteria, eukaryotes have signaling pathways designed to match the rates of translation and cell growth to intracellular nutrient and energy levels.

## 4

**mTOR and Amino Acids**

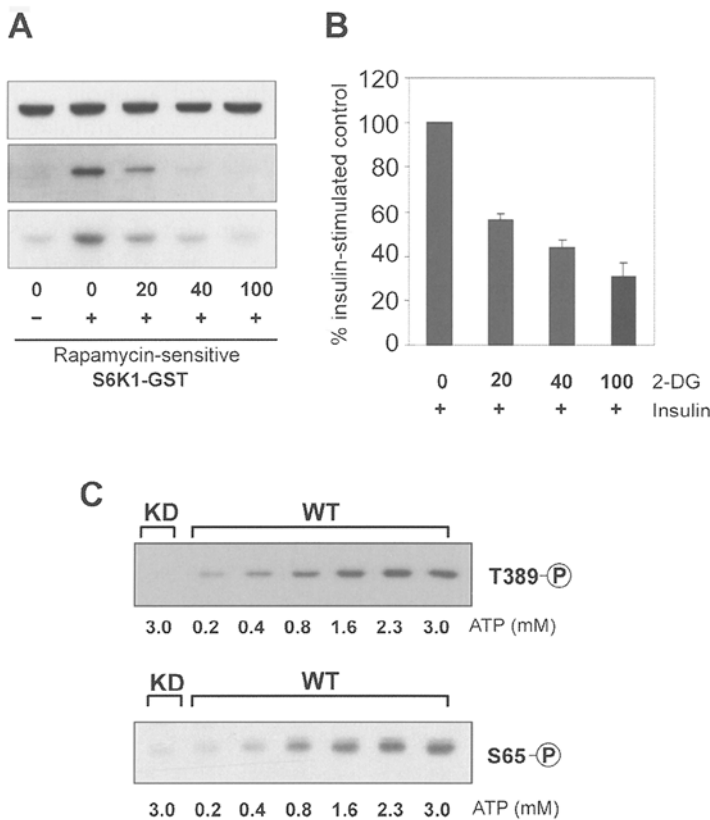
Amino acids have been shown to be obligatory for insulin signaling through mTOR (Hara et al. 1998). The mechanism by which amino acid deprivation attenuates mTOR function is not understood. Initially it was suggested that amino acid starvation leads to the accumulation of deacylated tRNAs, which in turn act to lower mTOR activity by a mechanism thus far unknown (Iiboshi et al. 1999). This hypothesis is based on observations in yeast where, upon amino acid deprivation, free histidyl tRNA accumulates and binds to a protein kinase termed GCN2, inducing its activation and the phosphorylation of initiation factor eIF2 $\alpha$  (Dong et al. 2000). Phosphorylation of eIF2 $\alpha$  acts to slow down translation and to increase the synthesis of GCN4, a transcriptional activator of over five hundred genes, a quarter of which encode for amino acid biosynthetic enzymes (Natarajan et al. 2001). However, the finding that amino acid deprivation for 30 min does not affect tRNA aminoacylation, while completely inhibiting S6K activation, supports the idea that amino acid pools rather than aminoacylated tRNAs are important for mTOR signaling (Dennis et al. 2001). Amino acid deprivation results in a decrease in the amounts of essential amino acids, particularly the branched-chain amino acids (Dennis et al. 2001). Of these, leucine has generated significant interest due to its unique ability to regulate S6K and 4E-BP at physiological levels, stimulate protein synthesis, and inhibit autophagy (Xu et al. 1998; Vary et al. 1999; Kimball et al. 1999). Indeed, incubation of cells with leucine in the absence of all the other amino acids rescues S6K activity (P.B. Dennis, A. Jaeschke, G. Thomas, unpublished data). This might be explained by the frequency of utilization of leucine in protein synthesis and by the existence of multiple leucyl-tRNA synthetases arising from a sixfold codon degeneracy. In addition, leucine not only functions as precursor for protein synthesis, but is also an allosteric activator of glutamate dehydrogenase (Plaitakis and Zaganas 2001) and a substrate for oxidative decarboxylation (Senner et al. 1980; Fahien et al. 1998), and therefore serves as mitochondrial fuel.

## 5

**mTOR and ATP**

Because mTOR has been implicated in ribosome biogenesis (Powers and Walters 1999; Mahajan 1994; Jefferies et al. 1994) and is sensitive to intracellular amino acid levels, the possibility is raised that it may also be sensitive to changes in intracellular levels of ATP. Consistent with such a model, lowering of intracellular ATP concentrations by treating cells with the glycolytic inhibitor 2-deoxy glucose leads to a dose-dependent inhibition of mTOR-mediated phosphorylation of S6K and a parallel decrease in intracellular ATP concentrations (Fig. 1; Dennis et al. 2001). Furthermore the specific activity of mTOR *in vitro* increases as ATP concentrations approach physiological levels using phosphorylation of S6K1 and 4E-BP1 as *in vitro* substrates (Fig. 1; Dennis et al. 2001). This finding can be explained by mTOR's apparent high  $K_m$  for ATP, which is 50- to 100-fold higher than the  $K_m$  described for most other protein kinases (Edelman et al. 1987). Such a high  $K_m$  would provide a potential regulatory mechanism whereby mTOR functions as a homeostatic sensor setting the rate of translation to the maximum allowed by the intracellular energy state. In mammalian cells ATP depletion does not affect amino acid pools, and amino acid deprivation has no effect on intracellular ATP levels, suggesting that both inputs use distinct mechanisms to control mTOR activity (Dennis et al. 2001). Unlike ATP, it should also be noted that free amino acids, including leucine, have no effect on mTOR activity *in vitro* (P.B. Dennis, A. Jaeschke, G. Thomas, unpublished data). Moreover, despite the fact that ATP and amino acids are necessary for mTOR signaling, they are not sufficient. They are both dependent on the growth promoting and metabolic actions of mitogens and hormones.

The effectiveness of glycolytic or mitochondrial inhibitors in different cell types depends on the pathway predominantly used to generate ATP. While in pancreatic  $\beta$ -cells mitochondrial inhibitors completely inhibit mTOR function, glycolytic inhibitors are more effective in transformed cells such as HEK 293 (Dennis et al. 2001; Xu et al. 2001). The effects appear specific for mTOR signaling, as the activities of other kinases such as PKB or MAPK are unaffected by such treatment (Dennis et al. 2001; Xu et al. 2001). By taking advantage of a rapamycin-resistant allele of S6K1, it was shown that the effects on S6K1 are through mTOR. Rapamycin resistance was conferred by fusing glutathione-S-transferase to the NH2 terminus of S6K1 and truncating the COOH terminus, creating



**Fig. 1** A Serum-starved cells transfected with S6K1-GST were extracted after insulin stimulation alone or in the presence of the indicated concentration of 2-deoxy glucose. B Mock-transfected HEK293 cells were treated with 2-deoxy glucose as in (A) and analyzed for total ATP using a luciferase-based assay kit, with results being expressed as a percentage of the insulin-stimulated control (Dennis et al. 2001). C HA-mTORwt (WT) or kinase dead (KD) were immunoprecipitated from transiently transfected, serum-starved HEK293 cells and assayed at the indicated ATP concentrations against S6K1 T389 or 4E-BP1 S65. (Reprinted with permission from Dennis et al. 2001)

a construct termed GST- $\Delta$ C-S6K1. This construct allows the monitoring of the phosphorylation and activation of S6K decoupled from mTOR, but still dependent on PI3K. Depletion of intracellular ATP did not affect insulin-induced activation of GST- $\Delta$ C-S6K1, supporting a model whereby mTOR is controlled by intracellular ATP concentrations (Dennis et

al. 2001). These effects may have been indirect, however, occurring through inhibition of tRNA aminoacylation. Support for this hypothesis comes from experiments showing that when amino acid alcohols such as leucinol or histidinol inhibit tRNA charging, amino acid-stimulated S6K1 activity is counteracted (Iiboshi et al. 1999). However, we demonstrated that neither insulin stimulation nor treatment with the glycolytic inhibitor 2-deoxyglucose had an effect on the total amount of aminoacylated tRNAs or on the levels of specific tRNAs, such as leucyl, histidyl, or threonyl tRNA (Dennis et al. 2001). Amino acid alcohols prevent tRNA charging by uncoupling the ATPase activity of the tRNA synthetases, which then may lead to constitutive consumption of ATP. The inhibitory effects of amino acid alcohols on mTOR signaling could thus be explained by ATP depletion in the presence of amino acid alcohols and mTOR inactivation. The observation that mTOR is regulated by energy may also help explain other seemingly controversial findings. For example, it has been noted that glucose regulates mTOR, but in some cells this has been difficult to demonstrate. Such discrepancies may reflect the ability of some cells to buffer their ATP levels with creatine phosphate.

## 6

### **Insulin Signaling Pathways**

While in single cell organisms, growth and proliferation are mainly regulated by nutrient availability, multicellular organisms require a complex signaling network to coordinate cell growth, proliferation, differentiation, and survival (Pfeiffer et al. 2001). Growth factors such as insulin regulate glucose and amino acid uptake and metabolism, and thus maintain ATP production and enable anabolic pathways (e.g., ribosome biogenesis and translation) required for cell growth. From studies in both the fly and mammalian tissues, mTOR is emerging as a critical effector for the integration of the metabolic and growth promoting functions of insulin (Dennis et al. 2001; Oldham et al. 2000; Xu et al. 1998; Zhang et al. 2000). Binding of insulin to the insulin receptor leads to activation and phosphorylation of the insulin receptor kinase, which then phosphorylates downstream signaling components including Shc, Gab-1, and Cbl/CAP, as well as the family of insulin receptor substrate (IRS) proteins on selective tyrosine residues. These phosphorylated residues serve as docking sites for downstream effector molecules. Binding of these

proteins to the insulin receptor triggers two major signaling cascades; the mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K) pathways (Zick 2001). Recruitment of the proteins Grb-2 and Sos to Shc activates the MAPK cascade, whereas association of PI3K with the IRS proteins results in production of phosphatidylinositol (Gingras et al. 2001; Powers and Walter 1999; Mahajan 1994)-trisphosphate (PIP<sub>3</sub>), which serves a second messenger in the activation of PKB, S6K, and atypical isoforms of PKC (Kozma and Thomas 2002). Together these kinase cascades mediate the metabolic and growth-promoting functions of insulin, such as the translocation of GLUT4 glucose transporter containing vesicles from intracellular pools to the plasma membrane, stimulation of glycogen and protein synthesis, uptake of amino acids and initiation of specific gene transcription programs (Zick 2001). The coordinate response triggered by insulin suggests that nutritional and mitogenic pathways have been integrated during evolution. One important element that seems to function as such an integrator of insulin action is mTOR. Unexpectedly, inhibition of mTOR function, either by treatment with rapamycin or by co-expressing dominant interfering alleles of mTOR, augments insulin-induced PI3K signaling (A. Jaeschke, P.B. Dennis, G. Thomas, unpublished data). In contrast, stimulation of mTOR function leads to attenuation of this signaling. Downregulation of the insulin-induced PI3K pathway by mTOR appears to be achieved in two ways. First by changing the subcellular redistribution of IRS1, which is thought to facilitate its proteosomal degradation; and second by increasing Ser/Thr phosphorylation, which reduces the affinity of the p85 regulatory subunit of PI3K for IRS1 and thereby uncouples IRS1 from PI3K (Zick 2001). Phosphorylation of IRS1 might be promoted by mTOR kinase activity directly or by an associated kinase (Hartman et al. 2001). Recently it has been demonstrated that PKC $\zeta$  and IRS1 form complexes in an insulin-dependent manner and that IRS1 is an *in vitro* substrate for PKC $\zeta$  (Ravichandran et al. 2001). It will be of interest to determine whether atypical PKCs play a role through mTOR in dampening insulin-induced PI3K signaling, similar to what has been described for the treatment of pseudo-adipocytes with rapamycin (Haruta et al. 2000). Atypical PKCs potentially may serve as adaptors in recruiting mTOR to IRS1 in an insulin-dependent manner and thereby regulating mTOR function. The negative feedback described above leads to downregulation of glucose uptake and other metabolic actions of insulin. As amino acid and energy levels decrease this will eventually lead to an inhibition



of mTOR signaling and cell growth. Thus with time, the impact of the negative feedback loop on PI3K will diminish, leading again to an increase in glucose and amino acid uptake. In this way it is hypothesized that mTOR acts to maintain tissue homeostasis as a function of insulin signaling and nutrient levels. The role of mTOR in maintaining tissue homeostasis is not limited to controlling signaling in the PI3K pathway; mTOR has also been shown to negatively regulate autophagy (Kamada et al. 2000), a process responsible for degradation of proteins and organelles during times of nutrient limitation, thereby generating amino acids, which can be used for energy (Dennis et al. 1999). Moreover, by decreasing the levels of proteins involved in anabolic processes, cellular metabolism also is downregulated in order to conserve energy. This ability to positively regulate ribosome biogenesis and protein synthesis, while negatively controlling protein degradation, supports a role for mTOR as a major intracellular integrator in mediating tissue and organ homeostasis.

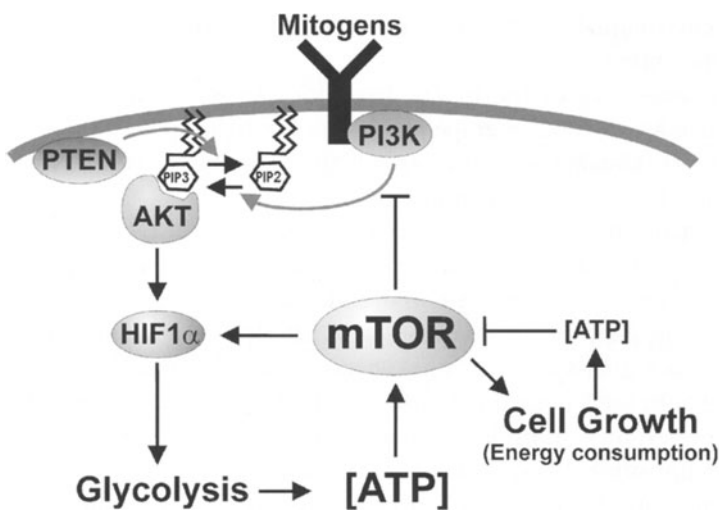
## 7

### **mTOR and Cancer**

Otto Warburg demonstrated more than 70 years ago that metabolic flux in many tumors is redirected to glycolysis (Warburg 1930; Dang and Semenza 1999), and in more recent studies it has been shown that oxygen limitation, e.g., in solid tumors or in the embryo during early embryogenesis (Shepard et al. 2000; Marsal 2002), leads to increased expression of genes that encode for glycolytic enzymes. The two major ATP-generating pathways are glycolysis and oxidative phosphorylation. Each pathway makes a different trade-off between yield and rate of ATP production. Although ATP can be rapidly generated by glycolysis, the yield is very low. Aerobic respiration on the other hand yields 10 times as much ATP, but at a much slower rate. Aerobic respiration is thought to have evolved with the advent of multicellular organisms as a means to better utilize energy resources (Pfeiffer et al. 2001). In an environment with limited resources, cells that use high rates of glycolysis will produce energy much faster but at a lower efficiency. By exploiting rate over yield, such cells will gain a growth advantage, since they more effectively compete for the fuel source and grow at a faster rate due to higher energy levels (Pfeiffer et al. 2001). The benefits of this growth advantage are augmented by the fact that in many solid tumors, cells exist in an oxy-

gen poor environment where oxidative phosphorylation is unable to function efficiently.

Recently, analogues of the mTOR inhibitor rapamycin have shown great promise as potential therapeutic agents for treating certain types of solid tumors (Hidalgo and Rowinsky 2000). A number of studies have indicated that tumor cells, depending on the mechanism of their evolution, can be particularly sensitive to rapamycin treatment (Neshat et al. 2001). One signaling element found mutated or absent in a number of glioblastomas (as well as prostate and endometrial tumors) is the tumor-suppressor gene PTEN (Neshat et al. 2001; Podsypanina et al. 2001). PTEN is a dual-specificity phosphatase that is capable of dephosphorylating both the 3 position of phosphatidyl 3,4,5 inositide and phospho-tyrosine residues on proteins (Maehama and Dixon 1999). It is the phosphatidylinositide phosphatase activity of PTEN that is important for the regulation of the proto-oncogene Akt/PKB. Akt is a multifunctional protein kinase that signals to cell survival pathways as well as metabolic and proliferation pathways (Hemmings 1997). Mutation or deletion of PTEN has been shown to result in aberrant activation of Akt leading to increased cellular metabolism and subversion of antitumor apoptotic programs (Yamada and Araki 2001). In an elegant set of experiments, Neshat et al. showed that when PTEN was deleted in isogenic MEFs or ES cell-derived tumors, the PTEN<sup>-/-</sup> cells gained a growth advantage that was selectively sensitive to treatment with the rapamycin analogue, CCI-779 (Neshat et al. 2001). In keeping with the mutator model of tumorigenesis (Loeb 2001), they further demonstrated that a prostate tumor cell line, which was initially resistant to rapamycin, became sensitive when these cells were stably transfected with a constitutively active form of Akt, i.e., when these tumors now arose as a function of having an active allele Akt, they became exquisitely sensitive to the effects of CCI-779 (Neshat et al. 2001). The link between the PTEN/Akt pathway and ATP production has been established in that constitutively active forms of Akt have been shown to increase levels of hypoxia-induced transcription factor 1 $\alpha$  (HIF1 $\alpha$ ; Jiang et al. 2001). Enzymes of the glycolytic pathway are major targets of the HIF1 $\alpha$ -mediated transcription necessary for redirecting metabolic flux when oxygen levels are low (Dang and Semenza 1999). Therefore, loss of PTEN may increase glycolytic flux through the activation of Akt and the increase of HIF1 $\alpha$ -mediated transcription. This increase in glycolytic flux would offset the loss of ATP due to the inhibition of oxidative phosphorylation at low oxygen



**Fig. 2** The model describes a potential mechanism by which Akt can drive increased mTOR signaling through raising ATP levels

levels and potentially give the cells a growth advantage that would utilize the mTOR pathway (see Fig. 2). Interestingly, similar models of PTEN loss of function are emerging in other disease states (Waite and Eng 2002). For example, loss of PTEN protein is associated with activation of synovial fibroblasts (Pap et al. 2000), which migrate and invade joints leading to destruction of cartilage and the advent of rheumatoid arthritis (Seemayer et al. 2001). The migration and proliferation of such cells are very sensitive to treatment with rapamycin (Migita et al. 1996).

8  
**Perspective**

The finding that the mTOR pathway is regulated by the level of intracellular ATP has prompted a reassessment of the mechanisms by which bioenergetics relates to signaling pathways known to be important for cell transformation. A tumor might be looked at as an evolutionary microcosm in which the combination of environment and genetic instability leads to the natural selection of cells with certain growth advantages. An mTOR-mediated growth advantage might occur over time as the progress of the tumor becomes more advanced and tumor cells overcome

the nutritional and hormonal constraints of their environments. In fact, PTEN mutants were originally identified as being enriched in advanced cancers. The idea that metabolic flux in tumors is redirected in a way that adapts a cell specifically to its environment is a concept that may lend itself to the elucidation of more targets and of more targeted anti-cancer therapies.

*Acknowledgments.* We would like to thank the members of the Thomas laboratory for their contributions to the studies described in this chapter. We also would like to thank the Deutsche Forschungsgemeinschaft (A. J.), Swiss Cancer League (P.B.D. and G.T.), and the Novartis Research Foundation (G.T.) for their support.

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# mTOR as a Positive Regulator of Tumor Cell Responses to Hypoxia

R. T. Abraham

Program in Signal Transduction Research, The Burnham Institute,  
10901 North Torrey Pines Road, La Jolla, CA 92037, USA  
*E-mail: abraham@burnham.org*

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**Abstract** Rapamycin is a clinically approved immunosuppressive agent that has recently shown promising antitumor activities in human patients. In contrast to many conventional chemotherapeutic agents, rapamycin displays a remarkably high level of selectivity for certain types of tumors. The pharmacological activities of rapamycin are attributable to the functional inhibition of a single target protein, termed the mammalian target of rapamycin (mTOR). Because mTOR is widely expressed in both normal and transformed cells, variations in mTOR expression levels are likely not a primary determinant of tumor sensitivity to rapamycin. However, recent studies highlighted an intriguing link between cancer cell sensitivity to rapamycin and deregulated signaling through the phosphoinositide (PI) 3-kinase pathway. These findings have prompted a search for cancer-related responses that are jointly regulated by the PI 3-kinase signaling cascade and mTOR. The oxygen-regulated transcription factor, hypoxia-induced factor (HIF)-1, has emerged as a candidate target for both of these two highly interactive signaling proteins. Here we review evidence that mTOR functions as a positive regu-



lator of HIF-1-dependent responses to hypoxic stress in human cancer cells.

## 1

### **A Family of PI 3-Kinase Related Kinases**

As reviewed in detail elsewhere (Abraham and Wiederrecht 1996), rapamycin is a bacterially derived macrolide ester whose mechanism of action in mammalian cells is exceptionally fascinating, but only partially understood. In order to exert any of its known cellular activities, rapamycin must first bind to a ubiquitously expressed intracellular receptor termed FKBP12. The resulting FKBP12-rapamycin complex functions as a potent and specific inhibitor of a high molecular mass (289 kDa) protein kinase termed mTOR by our laboratory (Sabers et al. 1995), and FRAP (Brown et al. 1994) or RAFT1 (Sabatini et al. 1994) by others. The primary amino acid sequence of mTOR sequence displays extensive homology with two budding yeast proteins (TOR1p and TOR2p), which were previously identified during screens for mutations that rendered yeast cells resistant to the antifungal activities of rapamycin (Cafferkey et al. 1993; Heitman et al. 1991; Kunz et al. 1993). The pharmacology of rapamycin in yeast, and the cellular functions of the yeast TOR proteins will be discussed in other chapters of this volume.

The mammalian and yeast TOR proteins are recognized as members of a novel superfamily of signaling proteins termed PI 3-kinase related kinases (PIKKs), based on conserved sequence motifs in their catalytic domains (Hunter 1995; Tibbetts and Abraham 2000). In spite of the sequence similarity to PI 3-kinases, the PIKKs that have been biochemically characterized to date phosphorylate protein rather than lipid substrates. However, protein kinase activity is not obligatory for membership in the PIKK family, since the yeast Tra1p and mammalian TRAPP proteins contain mutated versions of the PI 3-kinase-like catalytic domain that are predicted to lack phosphotransferase activity (Grant et al. 1998; McMahon et al. 1998). In these instances, evolution may have sacrificed intrinsic protein kinase activity in favor of molecular scaffolding functions for Tra1p and TRAPP during eukaryotic gene expression.

Mammalian cells express five kinase-active members of the PIKK family. Two of these protein kinases, ATM and ATR, serve as apical signal transducers in cell checkpoint pathways that orchestrate cellular responses to incompletely replicated or damaged DNA (Abraham 2001).

The third mammalian PIKK is the catalytic subunit of DNA-dependent protein kinase (DNA-PK<sub>cs</sub>), which functions primarily in DNA double strand break repair (Smith and Jackson 1999). The newest member of the mammalian PIKK family has been named ATX by our laboratory (manuscript submitted), and hSMG-1 by two other groups (Denning et al. 2001; Yamashita et al. 2001). Ongoing research suggests that ATX, and possibly ATM, reside at the crossroads of both DNA damage and RNA surveillance pathways. In accordance with their roles in DNA damage-induced signaling, ATM, ATR, and DNA-PK are localized largely in the nucleus, while ATX is found in both the nucleus and cytoplasm. Interestingly, these four “caretaker” PIKKs also share a notable biochemical property, i.e., a strong preference for the phosphorylation of Ser or Thr residues followed by Gln at the +1 position. A canonical phosphorylation site for these Ser/Thr-Gln-directed PIKKs is the conserved Ser-15 residue in p53, which is nested within a Leu-Ser-Gln-Glu sequence located at the p53 amino-terminus (Banin et al. 1998; Canman et al. 1998; Tibbetts et al. 1999).

In terms of its functions, localization, and phosphorylation site preference, mTOR displays some clear distinctions from the other members of the PIKK family. Although there is considerable disagreement on the subcellular localization of mTOR (for examples, see Kim and Chen 2000 and Desai 2002), we find that the protein is largely expressed in the cytoplasm in most tissue culture cells. Chen and coworkers have reported that mTOR undergoes constitutive nucleocytoplasmic shuttling (Kim and Chen 2000); however, we have found that this process is extremely slow, and its functional significance remains a mystery. In contrast to the ATM and DNA-PK subfamilies discussed above, neither mTOR nor its yeast counterparts play any direct roles in DNA damage responses. Instead, mTOR primarily responds to environmental cues, such as growth factors, amino acids, and glucose, and coordinates both cell growth and proliferation with the availability of these factors. Based primarily on studies in lower metazoan systems, it has been proposed that a major function of mTOR is to regulate the accumulation of cell mass, and that the inhibitory effects of rapamycin on cellular proliferation may result from activation of a “size checkpoint” that delays S phase entry until the cells have passed a critical threshold parameter related to cell volume (Montagne et al. 1999; Oldham et al. 2000; Stocker and Hafen 2000). Although this model is supported by strong genetic evidence from *Drosophila*, a recent report suggests the mechanisms that regulate over-

all body size are fundamentally different in insects versus mammals (Trumpf et al. 2001). Hence, whether mTOR functions primarily as a cell size regulator or a cell number regulator (or both) during mammalian growth remains an unsettled issue.

The protein kinase activity of mTOR will be reviewed in detail in other chapters of this volume. Briefly, mTOR exhibits no preference for the Ser/Thr-Gln motif favored by ATM, ATR, ATX, and DNA-PK, and correspondingly fails to phosphorylate the Ser-15 site in p53 described above. The most well-studied *in vitro* substrate for mTOR is the translational repressor protein, 4E-BP1/PHAS-I (Beretta et al. 1996; Brunn et al. 1997a, 1997b; Fadden et al. 1997; Gingras et al. 1999; Mothe-Satney et al. 2000; vonManteuffel et al. 1996). Although two of the known mTOR phosphorylation sites in human 4E-BP1/PHAS-I contain Thr followed by Pro at the +1 position, it would be inappropriate to apply the term “proline-directed kinase” to mTOR, as commonly used to describe MAP and cyclin-dependent kinases. At this point in time, a consensus site for phosphorylation by mTOR (if one exists) has not been defined.

## 2

### **Regulation of mTOR Signaling Functions**

The identities of the upstream signaling pathways that converge on mTOR, and the mechanisms through which they regulate mTOR function, remain elusive. Two mutually nonexclusive mechanisms have emerged as the leading candidates for the provision of afferent regulatory inputs into mTOR. Studies in yeast and flies indicate that mTOR is a component of a pathway that responds to nitrogen availability and/or amino acids (Oldham et al. 2000; Rohde et al. 2001; Schmelzle and Hall 2000). In mammalian cells, transfer into amino acid- and glucose-free medium appears to inactivate mTOR, and re-addition of certain amino acids, such as leucine, increases mTOR signaling, as measured by the activation of p70 S6 kinase and the phosphorylation of 4E-BP1/PHAS-I (Hara et al. 1998; Iiboshi et al. 1999; Rohde et al. 2001; Shigemitsu et al. 1999).

The proposed role of mTOR as a nutrient sensor in mammalian cells is now fairly well entrenched in the literature. However, investigators, both within and outside of the field, should pay heed to some unresolved issues before the proposed nutrient sensing function of mTOR enters the realm of dogma. First, it is highly questionable that the standard proto-

col (i.e., culture in amino acid-free medium) used to examine the “nutrient sensing” function of mTOR models any but the most extreme pathologic situations encountered by mammalian cells in tissues. Second, mammalian cell growth is not normally limited by nutrient supply, but rather by growth factor availability. In contrast, yeast cells grow and proliferate in a nutrient-controlled fashion as part of their normal life cycles. Therefore, it is logical that the growth-regulatory functions of the yeast TOR proteins would be linked to nutrient status. On the other hand, if we assume that mTOR plays important roles in the control of mammalian cell growth and proliferation, it is difficult to imagine that mTOR signaling functions are not modulated by the presence or absence of growth factors. The most parsimonious model posits that mTOR responds to both nutrient and growth factor availability, and the challenge at that this point is to define the biochemical pathways that link these distinct stimuli to mTOR in mammalian cells.

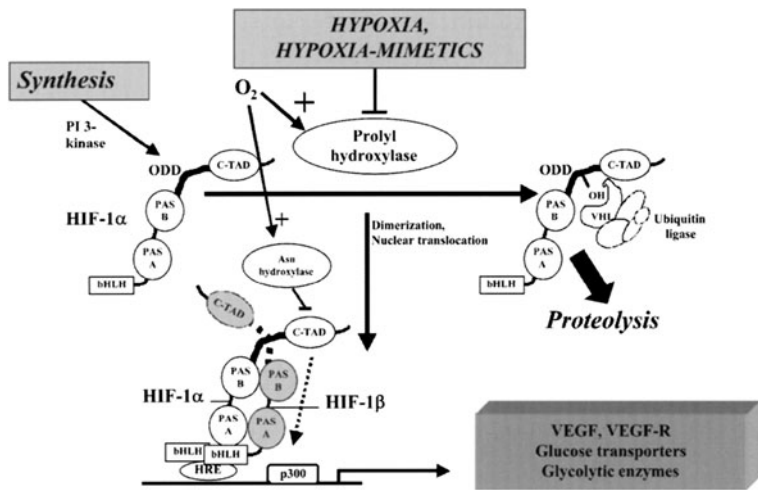
The growth factor receptor-triggered pathway that has garnered the most attention with respect to mTOR begins with the activation of PI 3-kinase. This enzyme is activated by a variety of growth factors, although certain classes of mitogens, including the insulin/insulin-like growth factors, seem more dependent on the PI 3-kinase signaling cascade than do others. PI 3-kinase activation leads to the accumulation of the multifunctional second messenger, phosphatidylinositol-3,4,5-trisphosphate (PIP<sub>3</sub>). This phosphoinositide binds to a host of cytoplasmic signaling proteins, in many cases through a PIP<sub>3</sub>-binding region termed the pleckstrin homology domain (Fruman et al. 1998; Katso et al. 2001). In general terms, the downstream targets for PI 3-kinase stimulate anabolic metabolism, cell growth and proliferation, and inhibit apoptosis. An important mediator of PI 3-kinase-dependent signals is the protein kinase, Akt (also termed PKB; Brazil and Hemmings 2001; Kandel and Hay 1999).

Considerable excitement has been generated by observations that mammalian cells bearing mutations that lead to deregulated signaling through PI 3-kinase/Akt show heightened sensitivities to the antiproliferative and anticancer activities of rapamycin (Mills et al. 2001; Neshat et al. 2001; Podsypanina et al. 2001). Clinical oncologists have taken particular notice of these studies, as they have a vested interest in the identification of molecular markers that might be predictive of rapamycin responsiveness in cancer patients. Furthermore, the emergence of *PTEN* as a major tumor suppressor gene product has abruptly elevated the PI 3-kinase pathway (and its suspected signaling partner, mTOR) to a star-

ring role in the process of tumorigenesis (Katso et al. 2001; Mills et al. 2001). After some confusion, the PTEN protein turned out to be a lipid phosphatase that specifically removes the D-3 phosphate from the *myo*-inositol ring of PIP<sub>3</sub> (Maehama and Dixon, 1999; Maehama et al. 2001; Simpson and Parsons, 2001). Loss of PTEN is a frequent event in many of the most lethal forms of cancer, including prostate cancer, glioblastoma multiforme, and malignant melanoma. Moreover, inactivation of *PTEN* alleles often occurs in late-stage tumors and correlates with increased proliferation and invasiveness, as well as with resistance to conventional chemo- and radio-therapeutic strategies.

The possibility that PTEN-deficient tumors have an Achilles heel—that is, heightened sensitivity to mTOR inhibition by rapamycin—has led to the suggestion that this drug has ushered in the long-awaited era of “tailored” molecular therapy for human cancers (Mills et al. 2001). For the time being, a very hopeful model is that aberrant PI 3-kinase signaling, whether caused by loss of PTEN, inappropriate signaling through insulin-like growth factor receptors, or other alterations, serves as a useful predictive marker for tumor responsiveness to rapamycin, as well as to other inhibitors of mTOR or Akt that are currently in the development stage.

In spite of the interest in the potential interplay between PI 3-kinase and mTOR, the evidence supporting this model is frustratingly incomplete. Several groups have reported that mTOR is a direct target for phosphorylation by Akt in mammalian cells (Nave et al. 1999; Scott et al. 1998; Sekulic et al. 2000). Interestingly, the phosphorylated Ser residue (Ser-2448) is located in a carboxyl-terminal region of mTOR that has been implicated in the regulation of mTOR kinase activity (Scott et al. 1998; Sekulic et al. 2000; see Fig. 1). The crucial piece of the puzzle that is missing at this point is the functional consequence of Ser-2448 phosphorylation with respect to mTOR signaling. Our results indicate that mTOR phosphorylation at Ser-2448 plays no role in the activation of p70 S6 kinase (Sekulic et al. 2000), a finding that has led some to conclude that Ser-2448 phosphorylation has no significance. However, it would seem prudent, for the time being, to apply the legal mantra, “absence of evidence is not evidence of absence,” as new insights into mTOR functions may well unveil the functional significance of mTOR phosphorylation by Akt.



**Fig. 1** Regulation of HIF-1 function by hypoxia. The synthesis of HIF-1 $\alpha$  is stimulated, at both the transcriptional and translational levels, by activation of the PI 3-kinase signaling pathway. The schematic depiction of HIF-1 $\alpha$  structural domains shows the basic helix-loop-helix (*bHLH*) domain and the PER-ARNT-SIM (*PAS*) domains, which mediate DNA binding and heterodimerization, respectively. The oxygen-dependent degradation (*ODD*) domain contains an amino-terminal transactivation domain (not shown). An additional, carboxyl-terminal transactivation domain (*C-TAD*) is found downstream of the *ODD* domain. Under normoxic conditions, newly synthesized HIF-1 $\alpha$  is rapidly modified by prolyl hydroxylases at two critical residues (Pro-402 and Pro-564) located in the *ODD* domain. The modified form of HIF-1 $\alpha$  is targeted for VHL-dependent ubiquitination and proteolysis. An asparagine hydroxylase simultaneously modifies and functionally inhibits the *C-TAD* in normoxic cells. Cellular exposure to hypoxia or hypoxia-mimetic agents (e.g., CoCl<sub>2</sub>) inhibits both amino acid hydroxylases, leading to the accumulation and transcriptional activation of the HIF-1 $\alpha$ ·HIF-1 $\beta$  heterodimer

3

**From Yeast to Mammals:  
Integration of mTOR into Nutrient Response Pathways**

As mentioned above, studies in yeast have documented a central role for the TOR proteins in the coordination of cell growth with the supply of nutrients. The adaptive response to limiting nutrient supply in yeast has been termed the “starvation phenotype” and represents a complex program of specific gene expression and repression, the goal of which is to allow the cells to survive the periods of famine that inevitably interrupt

those times when carbon and nitrogen sources are plentiful. On the other hand, bodily metabolism in mammals strives to maintain a stable flow of nutrients to the tissues during times of both feast and famine. The main vehicle for the delivery of vital nutrients to cells residing in the tissues is the blood. Hence, on what occasions do mammalian cells experience nutrient starvation in normal tissues?

One rather drastic example arises when the tissue blood supply is acutely interrupted—for example, during local hemorrhage or stroke. A second such situation, also pathological in nature, occurs when microtumors growing in tissues outstrip the local blood supply, and malignant cells, particularly those in the most interior regions of the tumor mass, are forced to cope with abnormally low levels of life-sustaining oxygen. Whether due to a cardiovascular event or tumorigenesis, tissue oxygenation almost invariably moves in lock-step with nutrient availability. A plausible hypothesis is that metazoan evolution has partially remolded the “primordial” nutrient response function of the TOR proteins to allow insertion of this signaling kinase into a stress-response program geared toward the maintenance of tissue homeostasis during periods of vascular insufficiency. This line of thinking leads very directly to a widely acknowledged “master” regulator of cellular responses to hypoxic stress—a heterodimeric transcription factor termed hypoxia-induced factor (HIF)-1. Ongoing research in several laboratories, including our own, is now forging ever-more compelling links between the PI 3-kinase/Akt/mTOR group of signal transducers and the transcriptional activity of HIF-1, particularly during the process of tumorigenesis.

Before launching in to a more detailed discussion of the interplay between mTOR and hypoxic adaptation, we need to consider in brief fashion the regulatory events that impinge on HIF-1, and the program of gene expression that is controlled by this oxygen-regulated transcription factor. For more detailed insights into HIF-1 regulation and function, the reader is referred to several excellent reviews (Semenza 1999, 2000, 2001).

The transcriptionally active form of HIF-1 is a heterodimeric complex typically comprised of HIF-1 $\alpha$  and HIF-1 $\beta$  subunits (Semenza 1999). Although we will not consider the possible contributions of alternative HIF-1 complexes to the hypoxic response program, it should be noted that mammalian cells variably express two additional HIF-1 $\alpha$ -related proteins (HIF-2 $\alpha$  and HIF-3 $\alpha$ ), which also pair with the HIF-1 $\beta$  subunit in hypoxic cells. The HIF- $\beta$  subunit is constitutively expressed in virtu-

ally all mammalian cells and bears the alternative name aryl hydrocarbon nuclear translocator (ARNT), based on its additional function as a partner for the  $A_h$  receptor, which mediates genotypic responses to certain xenobiotics, including the carcinogenic polycyclic aromatic hydrocarbons. The  $A_h$ -ARNT complex mediates genotypic responses to polycyclic aromatic hydrocarbons and other xenobiotics in mammalian cells. While the HIF-1 $\beta$  subunit is absolutely essential for HIF-1 function, it does not confer any known degree of oxygen sensitivity to the expression or function of the HIF-1 $\alpha$ -HIF-1 $\beta$  heterodimer. Rather, this critically important property of HIF-1 resides solely with the HIF-1 $\alpha$  subunit, and recent advances related to the control of HIF-1 $\alpha$  expression and function by oxygen have both surprised and fascinated a broad group of cell and molecular biologists.

As stated above, the HIF-1 $\beta$  subunit is continuously expressed in cells and tissues, regardless of the ambient oxygen tension. In contrast, most cells express very low levels of HIF-1 $\alpha$  under normoxic conditions, due in large part to the rapid degradation of newly synthesized protein via the ubiquitin-proteasome pathway (Fig. 1). In fact, a known tumor suppressor protein, von Hippel-Lindau (VHL), serves as the substrate-targeting subunit of a ubiquitin E3 ligase complex responsible for the rapid turnover of HIF-1 $\alpha$  in normoxic cells (Kondo and Kaelin 2001; Yang and Kaelin 2001). Patients with VHL disease display aberrantly elevated expression of HIF-1 $\alpha$  and are prone to the development of highly vascular tumors, including renal cell carcinoma and hemangioblastoma. The cancer-prone VHL syndrome underscores the notion that aberrant HIF-1 function is a positive factor during carcinogenesis. Under normal circumstances, however, the VHL-dependent ubiquitination pathway targets HIF-1 $\alpha$  for proteasome-mediated elimination with extremely high efficiency, as evidenced by the finding that the half-life of this protein is approximately 2 min in PC-3 prostate carcinoma cells maintained under standard culture conditions (our unpublished results). When these cells are switched to hypoxic conditions, the half-life of HIF-1 $\alpha$  abruptly and dramatically increases to over 2 h, leading to HIF-1 $\alpha$  accumulation. The protein then pairs with HIF-1 $\beta$ , and translocates to the nucleus to function as a nucleotide sequence-specific DNA binding protein.

The machinery that governs the oxygen-regulated turnover of HIF-1 $\alpha$  is now partially understood. HIF-1 $\alpha$ , like its partner HIF-1 $\beta$ , is a member of the basic helix-loop-helix family of transcriptional activators. In



addition to the usual DNA-binding and dimerization motifs, HIF-1 $\alpha$  contains a stretch of approximately 205 amino acids (residues 398–603) termed the oxygen-dependent degradation (ODD) domain. This region of the protein contains numerous serine and threonine residues, which prompted the logical prediction that phosphorylation-dephosphorylation cycles would govern the shifts in HIF-1 $\alpha$  stability observed at different oxygen tensions. Although phosphorylation events may well play a modulatory role in the regulation of HIF-1 $\alpha$  expression, the post-translational modification that directly oversees the targeting of HIF-1 $\alpha$  for rapid degradation was, until recently, far less familiar to even the most ardent signal transduction enthusiasts.

Elegant studies in *C. elegans* and mammalian cells have now uncovered a family of three HIF-1 $\alpha$ -directed prolyl hydroxylases (PHDs) that specifically modify HIF-1 $\alpha$  at two conserved prolyl residues (Pro-402 and Pro-564) residing in the ODD domain (Bruick and McKnight 2001; Epstein et al. 2001; Ivan et al. 2001; Masson et al. 2001; Semenza 2001). These enzymes require oxygen, ferrous iron (Fe<sup>2+</sup>), and oxoglutarate as cofactors. Under normoxic conditions, the PHDs avidly hydroxylate the ODD domain, and these modifications drive the recognition of HIF-1 $\alpha$  by the VHL-containing ubiquitin E3 ligase complex that mediates poly-ubiquitination of HIF-1 $\alpha$ . As the oxygen tension drops below 10%, the enzymatic activities of the PHDs decrease progressively, and unmodified HIF-1 $\alpha$  accumulates in the hypoxic cells. However, this remarkable mechanism of oxygen-sensitive regulation is not confined to the proline hydroxylation of HIF-1 $\alpha$  by the newly described PHDs. An equally novel, and as yet unidentified, amino acid hydroxylase modifies a critical Asn residue (Asn-851) located in the carboxyl-terminal activation domain (CAD) domain of HIF-1 $\alpha$  (Lando et al. 2002). This event inhibits the transcriptional activity of HIF-1 $\alpha$  (see Fig. 1). Once again, the activity of this Asn *N*-hydroxylase is inversely related to the oxygen tension, and modification of the CAD domain of HIF-1 $\alpha$  is dramatically decreased in cells exposed to hypoxic conditions. Thus, at least two sets of oxygen-sensitive amino acid hydroxylases act in concert to ensure that the expression of HIF-1-dependent genes is tightly coupled to those periods during which cells must cope with the stress imposed by a hypoxic microenvironment.

The complexity of the machinery that governs HIF-1 $\alpha$  accumulation and function raises many potential targets for modulation of this pathway by hormonal stimuli, environmental alterations, or internal cues,

such as those stemming from changes in cellular metabolism. For example, in spite of the general research emphasis on HIF-1 $\alpha$  stabilization, changes in the basal rate of HIF-1 $\alpha$  synthesis will inevitably affect the rate and level of HIF-1 $\alpha$  accumulation induced by hypoxia. Future research will undoubtedly uncover upstream signaling events that influence either the modification of HIF-1 $\alpha$  by the PHDs, or the recognition of hydroxylated HIF-1 $\alpha$  by the VHL-E3 ligase or other ubiquitin ligase complexes that use this protein as a substrate. As described below, the results emerging from several laboratories further tighten the linkage between the PI 3-kinase signaling cascade and HIF-1 $\alpha$  expression in both normal and malignant cells. Finally, a relatively under-explored area surrounds the post-translational events that govern the transcriptional activity of the HIF-1 heterodimer. Indeed, the Ras-Erk pathway has already been implicated as a mechanism to allow enhancement of HIF-1 transactivating function in mitogen-stimulated cells (Hur et al. 2001; Richard et al. 1999). Given the importance of HIF-1 to normal tissue maintenance, together with the pathologic sequelae of inappropriate HIF-1 activation, it is expected that multiple regulatory inputs will converge on this transcription factor in order to precisely tune the quantity and quality of HIF-1-dependent gene expression to the particular combination of oxygen availability and environmental cues extant in different tissue settings. The final section of this review summarizes recent evidence supporting the notion that mTOR serves as a positive regulator of HIF-1 function in hypoxic cells.

#### 4

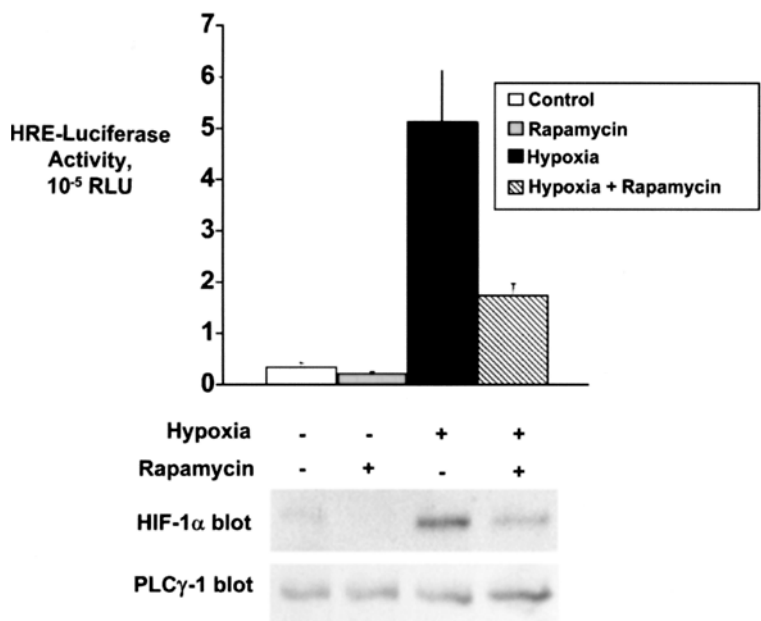
### **Emerging Roles of mTOR in the Regulation of HIF-1-Dependent Gene Expression in Hypoxic Cells**

The widespread availability of relatively specific pharmacologic probes, including wortmannin, LY294002, and rapamycin, made it a straightforward proposition to examine the roles of PI 3-kinase and mTOR in the activation of HIF-1 by hypoxia and stimuli. Giaccia and coworkers first showed that HIF-1-dependent gene expression in Ha-Ras-transformed fibroblasts was sensitive to inhibition by nanomolar concentrations of wortmannin, which is indicative of the involvement of PI 3-kinase (Mazure et al. 1997). In contrast, this response was unaffected by treatment of the cells with rapamycin, suggesting that mTOR was not a component of the signaling pathway leading to HIF-1 activation in the transformed

cells. Somewhat different results were later obtained by Semenza and coworkers, who studied hypoxia-induced HIF-1 activation in the human prostate cancer cell line PC-3 (Zhong et al. 2000). In agreement with the earlier findings, these investigators observed that pretreatment of PC-3 cells with a PI 3-kinase inhibitor (LY294002) strongly inhibited both the accumulation of HIF-1 $\alpha$  and the increase in HIF-1-dependent transcription induced by exposure to hypoxia or hypoxia-mimetic agents (e.g., CoCl<sub>2</sub>). However, Mazure et al. (1997), Zhong et al. (2000) found that these responses were also quite sensitive to an inhibitor of mTOR (rapamycin).

With the benefit of hindsight, these seemingly discrepant results should not have been so troubling, as rapamycin has been known for some time as a remarkably selective inhibitor of other cellular responses, such as proliferation (Hosoi et al. 1998). Moreover, we now have a much greater appreciation for the complexity of the regulatory events that impinge on HIF-1 $\alpha$  at both the transcriptional and post-transcriptional levels (Laughner et al. 2001; Semenza 1999, 2001). Based on these considerations, it seems reasonable to propose that the balance of signals leading to HIF-1 $\alpha$  accumulation in Ras-transformed fibroblasts downplays a role for the rapamycin target protein mTOR. On the other hand, the distinct intracellular signaling milieu in PC-3 cells dictates that the cascade leading to HIF-1 $\alpha$  is more heavily weighted toward mTOR, and hence more sensitive to rapamycin. Once again, we can invoke a unifying model in which loss of PTEN, and the resultant activation of PI 3-kinase signaling, renders the HIF-1 $\alpha$  induction pathway sensitive to rapamycin (Mills et al. 2001; Neshat et al. 2001; Podsypanina et al. 2001). As discussed earlier, the onus is on the proponents of this model to define the linkage between the PI 3-kinase/Akt pathway and mTOR during the activation of HIF-1 and other rapamycin-sensitive responses in mammalian cells.

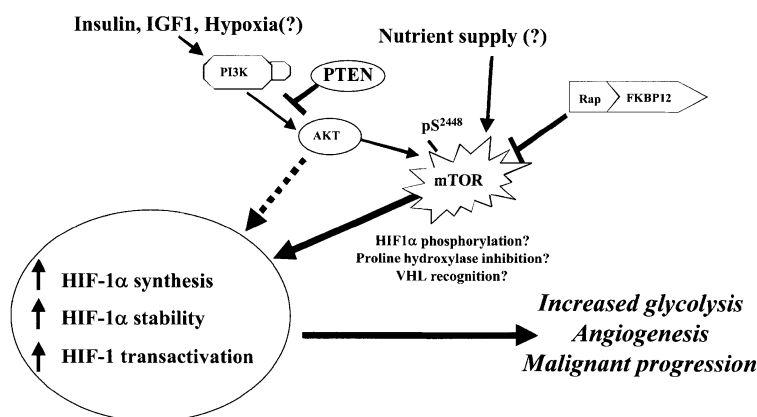
Our laboratory has recently followed up on the report by Zhong et al. (2000) in PC-3 cells. Our studies indicate that rapamycin treatment strongly but not completely inhibits hypoxia- or CoCl<sub>2</sub>-induced HIF-1 $\alpha$  accumulation in these cells (Fig. 2). Pulse-chase analyses indicated that the suppressive effect of rapamycin on the expression of HIF-1 $\alpha$  in hypoxic PC-3 cells is due in large part to a decrease in HIF-1 $\alpha$  stability, rather than to an inhibitory effect of the drug on HIF-1 $\alpha$  transcription or translation (Hudson et al. 2002). Furthermore, we observed that overexpression of wild-type mTOR amplified the increase in HIF-1-dependent gene expression triggered by hypoxic stress, while transfection of



**Fig. 2** Inhibition of HIF-1 $\alpha$  accumulation and HIF-1 function by rapamycin. *Upper panel:* PC-3 cells were transiently transfected with a luciferase reporter construct (pHRE-Luciferase) containing 5 tandem copies of the HIF-1 binding site from the human erythropoietin gene. The transfected cells were cultured for 8 h in 0.2% serum-containing medium, and then were exposed to hypoxia (1% O<sub>2</sub>) for 16 h, in the absence or presence of 100 nM rapamycin. Luciferase activities are reported as mean relative light units (RLU) from triplicate samples. *Error bars* indicate standard deviations. *Lower panel:* Whole cell extracts from the same samples were immunoblotted with  $\alpha$ -HIF-1 $\alpha$  antibody. Blots were stripped and re-probed with  $\alpha$ -phospholipase C (PLC)- $\gamma$ 1 antibody to control for sample loading in each lane

the cells with a rapamycin-resistant mTOR mutant, which contained a Ser<sup>2035</sup>→Iso substitution in the FRB domain (Brunn et al. 1997b; Stan et al. 1994), abrogated the inhibitory effect of the drug on hypoxia-induced HIF-1 activation. Although these responses should be extrapolated to other cell types with caution, the combination of pharmacologic and genetic data provide strong support for the conclusion that mTOR is a positive modulator of HIF-1 activation pathway in certain cellular contents.

The interactions between mTOR and HIF-1 are clearly not confined to the process of HIF-1 $\alpha$  stabilization in hypoxic cells (Fig. 3). Accumu-



**Fig. 3** The PI 3-kinase-Akt-mTOR signaling cascade as a pleiotropic amplifier of HIF-1 $\alpha$  accumulation in hypoxic cells. The working model proposes that PI 3-kinase and mTOR signaling positively affect HIF-1 function through stimulation of HIF-1 $\alpha$  synthesis, promotion of HIF-1 $\alpha$  stability, and enhancement of HIF-1 transcriptional activity

lating evidence suggests that the synthesis side of the HIF-1 $\alpha$  turnover equation is also positively affected by mTOR signaling in certain settings and cell types. Exposure of cells to specific polypeptide mitogens, such as epidermal growth factor, induces an increase in HIF-1 $\alpha$  protein, due in part to an increase in the steady-state level of HIF-1 $\alpha$  mRNA (Jiang et al. 2001; Laughner et al. 2001). As an aside, it is worth noting that growth factor stimulation appears to be far less efficient than hypoxia in terms of generating the transcriptionally active form of HIF-1 $\alpha$ , which is consistent with recent findings that an additional oxygen-sensitive modification regulates the function of the CAD of HIF-1 $\alpha$  (Lando et al. 2002). While growth factor- or hypoxia-stimulated *HIF-1 $\alpha$*  gene transcription is, at best, modestly suppressed by wortmannin, LY294002, or rapamycin, the results of several studies indicate that these drugs do affect HIF-1 $\alpha$  protein synthesis, particularly when this process is driven by hormonal stimuli (Jiang et al. 2001; Laughner et al. 2001). Therefore, it seems that the enhancement of HIF-1 $\alpha$  expression by these receptor-mediated stimuli occurs primarily at the post-transcriptional level.

Recent studies of the impact of rapamycin on heregulin (a HER2 receptor ligand)-induced HIF-1 $\alpha$  synthesis shed some important light on the mechanism of HIF-1 $\alpha$  induction by polypeptide mitogens (Laughner

et al. 2001). The authors demonstrated by pulse-chase analysis that HER2 receptor engagement triggered a striking increase in the rate of HIF-1 $\alpha$  protein synthesis, and that, in contrast to the heregulin-induced increase in HIF-1 $\alpha$  mRNA, this positive effect on translation was strongly suppressed by rapamycin. Interestingly, the 5'-untranslated region (UTR) of the HIF-1 $\alpha$  transcript contains several polypyrimidine tracts, which have previously been shown to strongly inhibit translation under conditions of growth factor or nutrient deprivation (Gingras et al. 2001). Moreover, these same sequence elements also confer strong translational dependence on PI 3-kinase and mTOR signaling. Whether these polypyrimidine tracts in the HIF-1 $\alpha$  mRNA actually control translational efficiency remains unclear; however, this working model meshes nicely with previous findings that deregulated PI 3-kinase signaling leads to amplified expression of HIF-1 $\alpha$  in hypoxic cells (Jiang et al. 2001; Zundel et al. 2000).

## 5

### Future Directions and Implications

While considerable progress has been made toward a complete understanding of the mechanism of action of rapamycin, some important gaps in the existing body of knowledge remain to be filled. First, accumulating evidence suggests that rapamycin may not inhibit mTOR signaling via direct interference with the protein kinase activity of this PIKK family member. If these findings are fully substantiated, then we will need to examine in detail the alternative model that binding of the FKBP12-rapamycin complex to mTOR disrupts the communication between mTOR and its upstream regulators or downstream target proteins. This scenario would also suggest that the effects of rapamycin on mTOR signaling functions are more subtle than previously anticipated, and could fuel discovery efforts aimed toward the identification of bona fide mTOR kinase inhibitors. One would expect that such inhibitors might display both quantitatively and qualitatively different pharmacologic activities than those observed with rapamycin.

Recent reports indicating a connection between mTOR and the cellular response to hypoxic stress raise some provocative questions related to both the basic biology of hypoxic adaptation, and the antitumor activities of rapamycin. First, the notion that the HIF-1 $\alpha$  mRNA is under the translational control of the PI 3-kinase-mTOR signaling pathway needs

to be confirmed in different cell types, and with different stimuli. One prediction from the existing data is that overexpression of eIF-4E in tumor cells would lead to enhanced HIF-1 $\alpha$  mRNA translation, and might represent one mechanism whereby malignant cells acquire resistance to rapamycin. Conversely, the sensitivities of certain tumors to rapamycin therapy *in vivo* might be explained by the inhibition of HIF-1-dependent gene expression in hypoxic cancer cells, a drug effect which could significantly compromise the abilities of these cells to cope with limiting supplies of oxygen and nutrients. Moreover, the panel of known HIF-1-inducible target genes includes several pro-angiogenic factors, including VEGF and its receptors (Elson et al. 2001; Semenza, 2000). Suppression of HIF-1 function in developing tumors during rapamycin therapy would therefore be expected to interfere with tumor-induced angiogenesis. In at least one tumor-host setting, this prediction was shown to be correct in a dramatic fashion, as neovascularization of an orthotopically implanted tumor mass was virtually abolished by treatment of the murine hosts with rapamycin (Guba et al. 2002).

In summary, studies of rapamycin and its target protein mTOR have unveiled many novel insights into mechanisms underlying cellular responses to growth factors and other environmental cues, including nutrients and oxygen. One has the distinct feeling that further functional analyses of mTOR will continue to yield new information regarding the signaling events that govern cellular metabolism, growth, and proliferation. Finally, rapamycin is already a clinically significant immunosuppressive agent, and its emerging clinical applications in the treatment of cancer and re-stenosis after angioplasty strongly hint that this drug will become one of the most significant therapeutic agents to hit the clinic in the past few decades. The clinical potential of rapamycin is almost certain to drive major pharmaceutical interest in the development of additional inhibitors of the mTOR-dependent signaling pathway. At the same time, rapamycin and its target protein are attracting the interest of an increasingly broad range of cell biologists, and we can be assured that the next few years will bring some major surprises regarding the regulation and functions of mTOR during both physiologic and pathologic processes in human tissues.

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# Retroviral Oncogenes and TOR

M. Aoki · P. K. Vogt

Department of Molecular and Experimental Medicine,  
The Scripps Research Institute, 10550 North Torrey Pines Road, BCC-239,  
La Jolla, CA 92037, USA  
*E-mail: pkvogt@scripps.edu*

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**Abstract** Retroviruses have recruited the catalytic subunit of PI 3-kinase and its downstream target, Akt, as oncogenes. These viruses cause tumors in animals and induce oncogenic transformation in cell culture. The oncogenicity of these viruses is specifically inhibited by rapamycin; retroviruses carrying other oncogenes are insensitive to this macrolide antibiotic. Rapamycin is an inhibitor of the TOR (target of rapamycin) kinase whose downstream targets include p70 S6 kinase and the negative regulator of translation initiation 4E-BP. Emerging evidence suggests that the TOR signals transmitted to the translational machinery are essential for oncogenic transformation by the PI 3-kinase pathway.

1

## Translational Controls in Cancer

Prevailing general ideas on oncogenesis focus on transcription, defining the neoplastic cellular phenotype as a consequence of deregulated pro-

duction of specific mRNAs. These ideas grew out of the abundance of transcriptional regulators that can function as nuclear oncoproteins and are currently further enhanced by the popularity of DNA microarrays and SAGE technologies in expression profiling. Although there is ample evidence for the importance of translational controls in cancer (for reviews see Rhoads 1991; Clemens and Bommer 1999; Zimmer et al. 2000), these data have been slow to enter mainstream thinking in cancer research. Yet as technological developments move toward a routine and comprehensive accounting of the proteome, control of translation will become more widely recognized as a critical factor in oncogenesis. This review follows the path from a cytoplasmic oncoprotein, activated PI 3-kinase, to its downstream targets, revealing unexpected effects on the regulation of protein synthesis. TOR emerges as an essential player in this oncogenic signal. There exist correlates to these experimental studies in human cancer; they establish mTOR and mTOR substrates as potential targets for novel cancer therapies.

## 2

### **Oncogenic PI 3-Kinase Signals**

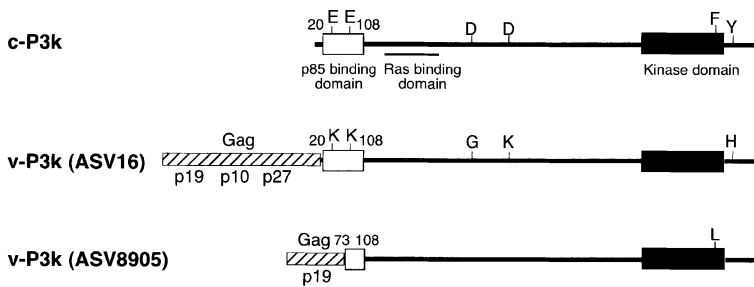
The heterodimeric enzyme PI 3-kinase (Class 1A) consists of a regulatory subunit p85, and a catalytic subunit p110 (for reviews see Katso et al. 2001; Vanhaesebroeck et al. 2001). The p85 subunit contains several protein recognition modules including SH2, SH3, and polyproline domains (Escobedo et al. 1991; Otsu et al. 1991; Skolnik et al. 1991). It receives upstream signals by interacting with receptor and non-receptor tyrosine kinases and activates the catalytic subunit p110 by translocating it to the plasma membrane (for reviews see Blume-Jensen and Hunter 2001; Wymann and Pirola 1998). PI 3-kinase phosphorylates phosphatidyl inositol at the D3 position (Whitman et al. 1988), generating phosphatidyl inositol-3,4-bisphosphate and phosphatidyl inositol-3,4,5-trisphosphate (for reviews see Fruman et al. 1998; Vanhaesebroeck et al. 2001). These products function as second messengers; they also serve to recruit important regulatory proteins, facilitating their interactions. PI 3-kinase affects multiple cellular functions related to growth, differentiation, and nutritional status; it forms an important nodal point that connects several signaling cascades and mediates crosstalk between these pathways (for reviews see Katso et al. 2001; Vanhaesebroeck et al. 2001).

The oncogenic potential of PI 3-kinase is documented by several observations. PI 3-kinase activities are associated via p85 with oncogene products such as polyoma middle T antigen and the viral Src protein (Fukui and Hanafusa 1989; Kaplan et al. 1986; Sugimoto et al. 1984; Whitman et al. 1985). The catalytic subunit of PI 3-kinase p110 can also be directly activated by the oncoprotein Ras (Rodriguez-Viciano et al. 1996). A constitutively active construct of p85 induces oncogenic transformation in collaboration with *v-raf* oncogene in mammalian cells (Jimenez et al. 1998). Two oncogenic avian retroviruses contain the coding sequences for p110 as oncogene (Aoki et al. 1998; Chang et al. 1997).

### 3

#### **The Avian Sarcoma Viruses ASV16 and ASV8905 Carry and Express a Mutant Form of p110**

Retroviral oncogenes are intron-less versions of cellular genes that code for components of signal pathways, and they are usually mutated. Two recently isolated retroviruses with oncogenic, cell-derived inserts in their genomes are ASV16 and ASV8905 (Aoki et al. 1998; Chang et al. 1997). Both code for homologs of the p110 subunit of class1A PI 3-kinase. These viral oncoproteins are referred to as viral P3k (v-P3k) in distinction to their cellular counterpart, c-P3k (p110). ASV16 and ASV8905 induce oncogenic transformation in avian embryo cell cultures, and both cause hemangiosarcomas when injected into young chickens. Maps of the viral genomes are shown in Fig. 1. A closer comparison of the v-P3k protein from ASV16 and c-P3k (p110) reveals significant differences. The viral P3k carries several amino acid substitutions and has suffered a 13-amino acid deletion at the amino terminus. The amino terminus of v-P3k is also fused to sequences of the viral Gag protein. These mutations in v-P3k could be responsible for strong oncogenicity of the viral P3k which stands in contrast to cellular P3k. Retrovirus-mediated expression of c-P3k, using the replication-competent RCAS vector that produces infectious progeny virus, is only poorly oncogenic in cell culture (Aoki et al. 2000). Such c-P3k-expressing constructs induce transformed cell foci with very low efficiency and only after extended latent periods. However, viral progeny harvested from these rare, transformed cell foci is highly oncogenic, comparable to v-P3k-expressing virus. This observation suggests a genetic change of c-P3k during passage as an insert in the retroviral RCAS vector. Analysis of the P3k protein in these



**Fig. 1** Schematic representation of cellular and viral P3k proteins (*c-P3k* and *v-P3k*). Amino acids that are different between the *c-P3k* and *v-P3k* proteins are marked. *v-P3k* occurs in two different avian sarcoma viruses, ASV16 and ASV8905. The *hatched boxes* represent retroviral Gag sequences. The *open box* marks the domain of PI 3-kinase that binds to the regulatory subunit p85, and the *black box* represents the catalytic domain of PI 3-kinase. The structures of the Gag-P3k fusion proteins differ in the two viruses; in ASV8905 the Gag portion is shorter, and the P3k has lost most of the p85-binding domain

transformed foci shows that each focus now produces an altered P3k that is fused to viral Gag sequences. The fusion points in individual foci of transformation vary, resulting in P3k-Gag proteins of different sizes. Each focus represents only one fusion event, generating transforming viral progeny that is clonal. Without this genetic change *c-P3k* is non-transforming (Aoki et al. 2000).

#### 4

#### Prerequisites for P3k-Induced Oncogenic Transformation

The correlation between fusion to viral Gag sequences and the activation of oncogenic potential suggests that the Gag domain provides a specific function needed in transformation. This suggestion was confirmed by engineering Gag-*c-P3k* fusions in vitro. Such constructs are highly oncogenic (Aoki et al. 2000). Since they contain the wild-type p110 coding sequence, the mutations in this sequence seen in the *v-P3k* proteins are unnecessary and irrelevant for oncogenicity. Retroviral Gag proteins can localize at the plasma membrane; in a Gag-*c-P3k* fusion, this membrane address could mimic membrane localization and activation as mediated by p85. Substituting the Gag sequences by a myristylation signal or attachment of a farnesylation signal at the carboxyl terminus of *c-P3k* also



yields transforming constructs and demonstrates the importance of a constitutive membrane address for P3k-induced oncogenicity. Such a membrane address makes activation by upstream signals that work through p85 redundant. Indeed, the v-P3k protein of ASV8905 has a 72-amino acid deletion at the amino terminus that removes more than half of the p85 interacting domain and can no longer bind to p85. Despite this inability to receive input from p85, the v-P3k of ASV8905 is highly oncogenic. Mutations that interfere with the binding of p110 to Ras also have no effect on oncogenicity, eliminating this interaction from the requirements for transformation. However, deletion of more than about 100 amino acids at the amino terminus and even small deletions at the carboxyl terminus of v-P3k destroy oncogenicity, and all of these deletions also abolish kinase activity of P3k. Since these deletions do not incur on the kinase domain, their effect must be indirect, possibly by inducing a conformational change in the protein that is incompatible with kinase activity. PI 3-kinase levels are elevated in P3k-transformed cells (Chang et al. 1997). Kinase-dead P3k fails to transform, and hence kinase activity is also essential for P3k-induced oncogenic transformation (Aoki et al. 2000).

## 5

### **Oncogenic PI 3-Kinase Signals Travel Through Akt**

The products of PI 3-kinase, phosphatidyl inositol-3,4-biphosphate and phosphatidyl inositol-3,4,5-triphosphate, are recognized by the pleckstrin homology domains of two serine-threonine kinases, Akt (also called PKB) and PDK1 (for reviews see Alessi and Cohen 1998; Alessi and Downes 1998; Chan et al. 1999; Coffey et al. 1998; Datta et al. 1999). Consequently, Akt and PDK 1 co-localize at the plasma membrane, and PDK 1 phosphorylates Akt on threonine 308, inducing Akt kinase activation (a second phosphorylation site, serine 473, may be targeted by a distinct activating kinase, PDK2). Like P3k, Akt has oncogenic potential. In fact, Akt was first discovered as an oncogenic cellular insert in the genome of the murine retrovirus AKT8 (Bellacosa et al. 1991). This mutated version of Akt induces hematopoietic malignancies in mice. It retains tumor-inducing activity when expressed in birds by the avian retroviral vector RCAS, but under these conditions Akt causes hemangiosarcomas that are indistinguishable from those seen with P3k (Aoki et al. 1998). Cellular Akt, carried and expressed by RCAS, is only marginally trans-

forming in cell culture. Attaching a myristylation signal to the amino terminus, or mutating the pleckstrin homology domain to enhance its affinity for the lipid products of PI 3-kinase, converts the innocuous cellular protein into a potent oncoprotein. These mutations of cellular Akt and the fusion to Gag sequences in the viral Akt again provide a constitutive membrane address which is a condition for transforming activity. The second prerequisite for Akt-induced oncogenicity is kinase activity. Mutating the PDK1 phosphorylation sites, threonine 308 and serine 473, to alanine abolishes both kinase activity and oncogenicity. A point mutation of lysine 179 to methionine at the ATP binding site in the kinase domain has the same effect. However, the phospho-mimetic mutations threonine 308 and serine 473 to aspartic acid fail to transform despite elevated kinase activity. Thus the requirements for oncogenicity of Akt are similar to those found with P3k, namely, membrane address and kinase activity.

The oncogenic signal from PI 3-kinase may therefore travel through Akt. Circumstantial evidence for this suggestion is provided by the close similarity of tumor types induced in birds by the two oncoproteins. Expression of the oncogenic versions of P3k but not of the non-oncogenic versions induces phosphorylation of serine 473 and hence activation of Akt (Aoki et al. 2000). A dominant negative mutant of Akt specifically interferes with P3k-induced transformation; it does not affect oncogenic transformation induced by other oncoproteins (Aoki et al. 1998). Akt is therefore an essential component of the oncogenic signal issued by constitutively active PI 3-kinase.

## 6

### **Transformation-Relevant Targets of Akt**

Gain of function in PI 3-kinase or Akt causes oncogenic transformation; both oncoproteins belong to the same signaling pathway. The direct or indirect downstream targets of Akt that could be important mediators of oncogenicity are listed in Table 1. Several pro-apoptotic proteins as well as GSK-3 $\beta$  are downregulated by Akt (for reviews see Alessi and Cohen 1998; Chan et al. 1999; Datta et al. 1999; Downward 1998). The growth-attenuating forkhead-winged helix transcription factors FKHR, FKHR-L1, and AFX are also downregulated by exclusion from the nucleus when they become phosphorylated by Akt (Biggs et al. 1999; del Peso, et al. 1999; Guo, et al. 1999; Nakae, et al. 1999; Rena, et al. 1999; Tang, et

**Table 1** Targets of Akt**Target**

Survival and death factors  
 Bad  
 Caspase 9  
 Ask1  
 Cell cycle, cell proliferation  
 FKHR, FKHR-L1, AFX  
 IKK $\alpha$   
 p21<sup>CIP-1/WAF-1</sup>  
 Mdm2  
 GSK-3  
 Raf-1  
 Translational machinery  
 mTOR  
 p70<sup>S6K</sup> (S6K)  
 4E-BP1

**Function**

Pro-apoptotic BH3 protein, counteracts Bcl-xL  
 Pro-apoptotic protease  
 Pro-apoptotic MAPKKK, regulates JNK and p38  
 Growth inhibitory transcriptional activators  
 Serine/threonine kinase, inhibitor of NF $\kappa$ B  
 CDK inhibitor  
 Ubiquitin ligase, regulates p53 stability  
 Serine/threonine kinase with multiple functions  
 Oncogenic MAPKKK, regulates Erk pathway  
 Serine/threonine kinase, regulates S6K and 4E-BP1  
 Serine/threonine kinase, phosphorylates the S6 ribosomal protein  
 Inhibitor of eukaryotic initiation factor 4E

al. 1999; Brunet et al. 1999; Kops et al. 1999; Takaishi, et al. 1999). The combined effects of all these target modifications would result in increased cell survival. The Akt-dependent phosphorylation of IKK $\alpha$  ultimately results in the stimulation of NF $\kappa$ B activity (Kane et al. 1999; Ozes et al. 1999; Romashkova and Makarov 1999), likely to inhibit apoptosis and enhance cell growth. The list also contains the mammalian target of rapamycin mTOR (for reviews see Gingras et al. 2001; Dennis et al. 1999; Schmelzle and Hall, 2000; Dennis and Thomas 2002) and two mTOR-regulated proteins, p70 S6 kinase (S6K) and 4E-binding protein (4E-BP). The latter two have important functions in the initiation of protein syn-

thesis. S6K activity is required for translation of the 5'TOP class of mRNAs that code for components of the translational machinery (Jeffries et al. 1997). The protein 4E-BP controls availability of the eukaryotic initiation factor eIF4E, the limiting component for the formation of the 4F cap-binding complex (Brunn et al. 1997; Burnett et al. 1998; Gingras et al. 1998, 1999a). Availability of eIF4E selectively affects mRNAs with complex secondary structure in their 5' untranslated region (for reviews see Gingras et al. 1999b; Sonenberg and Gingras 1998). Many of these code for growth-regulatory proteins. The list of Akt targets allows for diverse mechanisms of oncogenic transformation that include transcriptional controls as well as translational controls. Transcriptional controls probably play a part in P3k- and in Akt-induced oncogenicity. For example, co-expression of the I $\kappa$ B superrepressor (inhibitor of NF $\kappa$ B activation) results in smaller foci of cells transformed by P3k and Akt (M. Aoki and P.K. Vogt, unpublished data).

## 7

### The Oncogenic Signal from PI 3-Kinase Requires TOR

The importance of translational controls in P3k- and in Akt-induced transformation was revealed by a high sensitivity of these oncoproteins to rapamycin (Table 2; Aoki et al. 2001). Rapamycin interferes at low concentrations specifically with transformation induced by P3k or by Akt but does not affect a wide variety of other oncoproteins. Some oncoproteins, notably Ras and Myc, are even enhanced in their transforming activity by rapamycin, but this effect appears to be restricted to avian fibroblasts. Rapamycin not only prevents the induction of transformed cell foci by P3k and by Akt, it also reverts the P3k- or Akt-transformed phenotype into that of normal cells; but again has no effect on the phenotype of cells transformed by other oncoproteins (A. Bader and P.K. Vogt, unpublished data).

Rapamycin interacts with the cellular protein FKBP12, and this complex then binds to and inactivates TOR, thereby affecting the downstream targets 4E-BP and S6K (for review see Gingras et al. 2001). Active TOR induces phosphorylation of 4E-BP and of S6K; this phosphorylation activates S6K and inactivates 4E-BP. Hyperphosphorylated 4E-BP dissociates from its partner eIF4E, setting eIF4E free to join the cap-binding complex that initiates translation (Brunn et al. 1997; Burnett et al. 1998; Gingras et al. 1998; Gingras et al. 1999a). In cells transformed

**Table 2** Effect of rapamycin on transforming activities of various oncogenes in chicken embryo fibroblasts

Oncoprotein	Rapamycin (1 ng/ml) <sup>a</sup>
Group 1: Inhibited	
Myr-c-P3k	>0.01
Myr-Akt	>0.01
Group 2: Unaffected	
v-Src	1.00
v-Yes	0.75
v-Sea	0.58
v-Abl	1.00
v-Fps	1.02
v-ErbA/v-ErbB	1.40
v-Crk	0.78
v-Mos	1.17
v-Jun	0.92
v-Fos	1.17
Group 3: Enhanced	
v-Myc	10.40
v-Myc/v-Mil	16.00
v-H-Ras	14.00
v-Maf	2.40

<sup>a</sup> Efficiency of transformation: number of transformed cell foci in the presence of rapamycin over number of transformed cell foci in the control.

by P3k or Akt, S6K and 4E-BP are constitutively phosphorylated, making S6K active and abolishing the inhibitory effect of 4E-BP on translation initiation. Constitutive phosphorylation of these two regulatory components of protein synthesis is not seen with a kinase-active but non-transforming mutant of Akt (Aoki et al. 2001). The phosphorylation of S6K and 4E-BP is inhibited by rapamycin in an FKBP12-dependent manner, indicating that the effect of rapamycin is a consequence of TOR inhibition. These data show TOR activity is essential for P3k- and Akt-induced oncogenicity but not for transforming activity of other oncogenes. The correlation of constitutive phosphorylation of S6K and of 4E-BP with transforming activity also suggests that these two regulatory, TOR-controlled proteins play an important role in the transformation process. Even though other oncoproteins are not affected by rapamycin alone, some, e.g., Src, can be inhibited by a combination of rapamycin and in-

hibitors of the MAP kinase pathway (Penuel and Martin 1999). In addition, transformation of an immortalized rat kidney epithelial cell line by the zinc finger transcription factor Gli but not by Ras or Myc was also shown to be rapamycin-sensitive (Louro et al. 1999). TOR and its regulation of protein synthesis may therefore play a much broader role in oncogenic transformation than is suggested by the analysis of P3k and Akt.

## 8

### Protein Synthesis and Cancer

There are multiple indications suggesting that components of the protein synthesizing machinery have oncogenic potential. The eukaryotic initiation factors 4E, 4G, and a mutant of 2A and 3e transform certain cell types (De Benedetti and Rhoads 1990; Lazaris-Karatzas et al. 1990; Fukuchi-Shimogori et al. 1997; Donze et al. 1995; Mayeur and Hershey 2002). A truncation mutant of S6K can cause a partially transformed phenotype (Mahalingam and Templeton 1996). Hyperphosphorylation and thus inactivation of 4E-BP is seen in cells transformed by the Src oncoprotein (Tuhackova et al. 1999) and in the cells of PTEN  $-/-$  mice (Neshat et al. 2001). Although the oncogenic potential of P3k and of Akt was first recognized in studies of transforming retroviruses, the relevance of all these kinases extends to human cancer. PIK3CA, the catalytic subunit of PI 3-kinase in the human genome, is amplified and overexpressed in ovarian cancer (Shayesteh et al. 1999). The three *AKT* genes of the human genome show gain of function in diverse tumors, including gastric, ovarian, breast, pancreatic, and prostate cancers (Bellacosa et al. 1995; Cheng et al. 1992, 1996; Miwa et al. 1996; Nakatani et al. 1999; Ruggeri et al. 1998). The eukaryotic initiation factor 4E is overexpressed in lymphomas, cancers of the head and neck, and in cancers of the colon (De Benedetti and Harris 1999). The phosphatase antagonist of PI 3-kinase, PTEN, is an important tumor suppressor and is inactivated in many human cancers, notably cancer of the prostate and glioblastoma (Li et al. 1997, for reviews see Ali et al. 1999; Cantley and Neel 1999; Maehama and Dixon 1999). In prostate cancer cell lines, inactivating mutations of PTEN or gain-of-function mutations of Akt are correlated with constitutive phosphorylation of mTOR (Sekulic et al. 2000). A recurrent finding in expression profiles of cancer cells is the upregulation of mRNAs that code for ribosomal proteins, suggesting involvement of S6K (Ross et al. 2000; see also the website of the Cancer Ge-

nome Anatomy Project of the National Cancer Institute: <http://cgap.nci.nih.gov/>). S6K is amplified and overexpressed in breast cancer cell lines as well as in primary breast tumors (Barlund et al. 2000; Couch et al. 1999). Recent publications have drawn attention to the important role of mTOR in PTEN negative and in Akt-overexpressing tumors. PTEN<sup>+/-</sup> mice show an increased incidence of various tumors including those of the uterus and the adrenal medulla (Di Cristofano et al. 1998; Podsypanina et al. 1999; Suzuki et al. 1998). In these tumors, PTEN levels are greatly decreased and S6K is activated, as judged by its phosphorylation status and by immune complex kinase assays (Podsypanina et al. 2001). Treatment of mice with the rapamycin analog CCI-779 causes a reduction in S6K activation and inhibits tumor growth (Podsypanina et al. 2001). In PTEN-negative human tumor cell lines, total and phosphorylated levels of the ribosomal protein S6 (the substrate of S6K) are increased, and 4E-BP is mostly phosphorylated (Neshat et al. 2001). CCI-779 reduces S6 and 4E-BP phosphorylation. Tumor formation by PTEN-negative or by Akt-overexpressing human cancer cells in SCID mice is also inhibited by CCI-779 (Neshat et al. 2001). The effect of CCI-779 on tumorigenesis may at least in part derive from antiangiogenic activity (Guba et al. 2002). Rapamycin downregulates VEGF at the transcriptional level and probably also at the translational levels. Translation of VEGF is highly dependent on 4E (Kevil et al. 1996; Scott et al. 1998).

The rapamycin-mTOR connection clearly implicates deregulated protein synthesis in cancer. mTOR and its targets play an essential role in those cancers that are driven by a gain of function in PI 3-kinase signaling. The aspects of protein synthesis that are affected by these oncogenic signals are translation of 5'TOP mRNAs and of mRNAs with complex 5' untranslated structures requiring extensive unwinding by the 4F initiation complex. Deregulation of these messages would lead to increased capacity of the protein-synthesizing machinery and to elevated amounts of growth stimulatory, regulatory proteins. It is not clear at this time whether enhanced translation of 5'TOP or of 4E-dependent mRNAs alone can lead to oncogenic transformation or whether both types of messages have to be affected. The involvement of TOR in oncogenic transformation raises several questions: (1) TOR is necessary for oncogenicity, but is it sufficient? Can a gain of function in TOR lead to transformation? (2) What are the critical targets selectively affected by the availability of the eukaryotic initiation factor 4E? Can overexpression of such a target induce resistance to rapamycin? (3) What is the molecular

mechanism for the selective effect of 4E abundance on certain mRNAs? Are specific RNA structures responsible for the selectivity, and what are the defining criteria of these structures?

Questions also remain on the mechanism by which TOR regulates S6K and 4E-BP. A linear signal chain from PI 3-kinase via Akt and TOR to S6K is likely an oversimplification (Raught et al. 2001). A recent study has identified a conserved TOR signaling motif (TOS) in both S6K and 4E-BP (Schalm and Blenis 2002). TOS is a stretch of five amino acids located in the amino terminal region of S6K. It mediates two mechanistically distinct TOR-dependent activation steps of S6K, one involving phosphorylation of threonine 389, the other abrogation of an inhibitory activity that maps to the carboxyl terminal region of S6K. A better understanding of TOR function will also have to take into account other players in this arena, such as the tumor suppressors of the TSC complex (Kwiatkowski et al. 2002; Montagne et al. 2001) and the regulation of a PPA-like phosphatase (Dufner and Thomas 1999; Hartley and Cooper 2002; Inui et al. 1998; Murata et al. 1997). The function of nucleocytoplasmic shuttling of TOR also needs to be elucidated (Kim and Chen 2000). It is clear that we are only at the beginning of the exciting and challenging task of exploring the full spectrum of TOR functions. It is equally clear that these investigations will generate insight and reveal promising targets for drug development.

*Acknowledgments.* Work of the authors is supported by grants from the National Cancer Institute, National Institutes of Health. This is manuscript number 14932-MEM at The Scripps Research Institute.

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# mTOR as a Target for Cancer Therapy

P. J. Houghton · S. Huang

Department of Molecular Pharmacology, St. Jude Children’s Research Hospital,  
332 N. Lauderdale, Memphis, TN 38105–2794, USA

E-mail: *peter.houghton@stjude.org*

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**Abstract** The target of rapamycin, mTOR, acts as a sensor for mitogenic stimuli, such as insulin-like growth factors and cellular nutritional status, regulating cellular growth and division. As many tumors are driven by autocrine or paracrine growth through the type-I insulin-like growth factor receptor, mTOR is potentially an attractive target for molecular-targeted treatment. Further, a rationale for anticipating tumor-selective activity based on transforming events frequently identified in malignant disease is becoming established.

## 1

**Introduction**

Since the early 1960s the introduction of cytotoxic agents, thence their dose intensification (starting in the 1970s), has dramatically improved survival of children with hematologic and solid tumors. For example, data from 1960–1963 shows that overall survival for children under fifteen years old with a diagnosis of neuroblastoma, bone or joint sarcomas, or CNS tumors was 25%, 20%, and 35%, respectively. In contrast, for the period 1985–1994 the survival for these same groups had increased to 63%–65%. However, for soft tissue sarcomas, the focus of this laboratory, increases in survival have been less impressive, increasing from 60% in 1974 to 71% (1985–1994)<sup>1</sup>. Although these results demonstrate clear progress in treating childhood malignancies, they do not reflect the morbidity and long-term sequelae often associated with intensive use of cytotoxic agents. Consequently, almost two decades ago we started to study pediatric soft tissue sarcomas with the ultimate goal of developing novel therapeutic approaches based on specific biological characteristics of these tumors. In this chapter we will review the process that allowed us to stumble onto rapamycin as a potential therapeutic agent, and the progress in understanding why this macrolide antibiotic may exert tumor-specific cytotoxicity.

## 2

**Autocrine Growth of Rhabdomyosarcomas**

Our laboratory has focused on rhabdomyosarcoma, a tumor of skeletal muscle origin, and in particular a particularly aggressive “alveolar” variant thereof. Cytogenetic analysis of several of these tumors from independent patients that were established as xenografts in immune-deprived mice showed a consistent chromosomal translocation t(2;13) (q35;q14; Hazelton et al. 1987). A more systematic survey of patient tumor biopsies demonstrated consistent translocations in greater than 90% of alveolar rhabdomyosarcomas (Douglass et al. 1987). We now know that this translocation results in expression of a chimeric tran-

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<sup>1</sup> Years 1974 to present based on SEER data [Ries LAG, Miller BA, Guernsey JG, Linet M, Tamra T, Young JL, Bunin GR (eds)]. SEER Program, 1975–1995; Tables and Graphs, National Cancer Institute. NIH Pub. No. 99–4649. Bethesda, MD, 1999



scription factor (PAX3/FKHR) that appears to block myogenic differentiation (Galili et al. 1993; Shapiro et al. 1993; Epstein et al. 1995) leading to tumor formation. These studies lead to further characterization of alveolar rhabdomyosarcoma cell lines, and revealed overexpression of transcripts for type II insulin-like growth factor (IGF-II) specifically from the fetal P3 promoter. In collaboration with Lee Helman at the Pediatric Branch, National Cancer Institute (NCI), Bethesda, we were able to show that growth of rhabdomyosarcoma cells was driven by an autocrine loop. Specifically, cells secreted IGF-II, and signaled through the IGF-I receptor (El-Badry et al. 1990). Inhibition of the IGF-I receptor by using a neutralizing antibody inhibited tumor cell growth. This suggested to us that interference with IGF-I-receptor-mediated signaling may be a therapeutic strategy for these tumors. To test this concept, the IGF-I receptor was downregulated using a stable expression of antisense constructs (Shapiro et al. 1994). These studies showed a high correlation between downregulation of the receptor, decreased growth in soft agar (such growth being a characteristic of malignant cells), and decreased formation of tumors when cells were inoculated into immune-deprived mice. Further, clones with the lowest expression of IGF-I receptor expressed the highest levels of the myogenic marker, MyoD, and formed multinucleate syncytia, thus recapitulating some characteristics of myogenic differentiation. While our studies focus on rhabdomyosarcomas, there is increasing evidence that many tumors are "driven" by either autocrine or paracrine signaling through the IGF-I receptor. Clearly deregulated IGF-I signaling is frequent in many pediatric solid tumors (neuroblastoma, Ewing's sarcoma, Wilms' tumor, medulloblastoma, glioblastoma), as well as many adult carcinomas (Macaulay, 1992; Toretsky and Helman 1996). Direct inhibition of the IGF-I receptor with antibody, while effective in mouse models (Kalebic et al. 1994), was not at that time plausible in humans due to antigenicity, although this is now less of a problem and such reagents are being developed for clinical application. Alternatively, we sought a small molecule inhibitor of IGF-I signaling.

### 3

#### **Selective Tumor Growth Inhibition by Rapamycin**

Our studies with rapamycin started in 1992, and were stimulated by a chance conversation with Randall Johnson at SmithKline Beecham. At that time it was known that the immunosuppressive agents FK506 and

**Table 1** Sensitivity of childhood rhabdomyosarcoma cell lines and human colon carcinoma cell lines to rapamycin and geldanamycin

	Rapamycin IC <sub>50</sub> (ng/ml)	Geldanamycin C <sub>50</sub> (nM)
Rhabdomyosarcoma cell lines		
Rh1	4,680	5.9
Rh18	0.1	14.3
Rh28	8.0	17.9
Rh30	0.37	1.9
Colon carcinoma cell lines		
GC <sub>3</sub> /c1	9,800	3.6
VRC <sub>5</sub> /c1	1,280	1.4
CaCo	1,570	3.4
HCT8	8,400	ND <sup>a</sup>
HCT29	>10,000	2.6
HCT116	>10,000	ND
National Cancer Institute screen (60 cell lines)	3,160	

<sup>a</sup> ND, not determined (from Dilling et al. 1994).

cyclosporin A blocked T cell activation prior to expression of interleukin-2, whereas rapamycin acted downstream of interleukin-2 expression (Flanagan et al. 1991; Schreiber and Crabtree 1992; McCaffrey et al. 1993). There was also some suggestion that for T cells to progress to S phase, the IGF-I receptor had to be expressed (Reiss et al. 1992). We speculated that perhaps rapamycin acted downstream of the IGF-I receptor to block cell cycle progression, and if so, may act to inhibit the growth of rhabdomyosarcoma cells. The results, shown in Table 1, were both surprising and exciting. Three of four rhabdomyosarcoma cell lines were exquisitely sensitive to rapamycin whereas one line, (Rh1), which is less dependent on IGF-I mitogenic signaling, was highly resistant. The other interesting aspect of these results was the marked selectivity for rhabdomyosarcoma cells relative to colon carcinoma cells, or cells used in the NCI in vitro screen (Dilling et al. 1994). Intriguing, but not comprehended (at that time) was the observation that under serum-free conditions Rh1 cells became very sensitive to rapamycin, with the IC<sub>50</sub> decreasing from 5,800 ng/ml to 3.6 ng/ml. Consistent with results from other laboratories, the effect of rapamycin could be competed using FK506, indicating that initial formation of the FKBP-rapamycin complex was important.

## 4

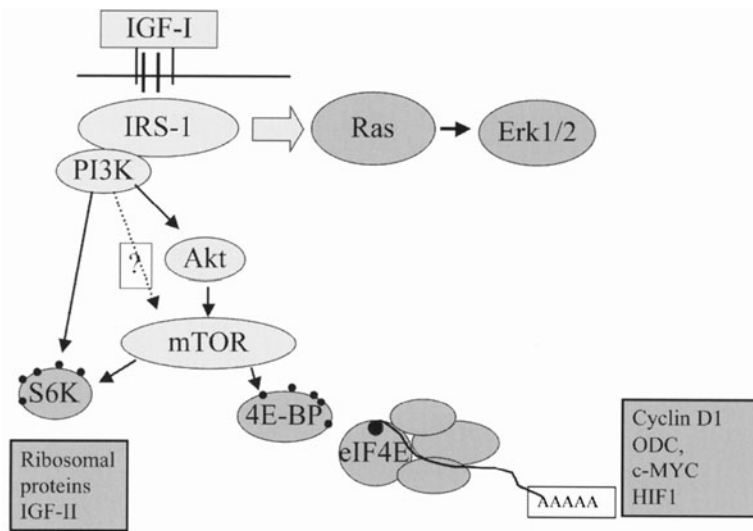
**Mechanism of Action of Rapamycin**

The action of rapamycin will be covered in detail elsewhere. Briefly, rapamycin first binds the immunophilin FKBP-12 (the 12-kDa FK506 binding protein) and this complex is now known to be a specific inhibitor of a serine/threonine kinase mTOR (the mammalian target of rapamycin, also called FRAP/RAPT/RAFT; Brown et al. 1994; Sabatini et al. 1994; Chiu et al. 1994; Sabers et al. 1995). Kinase mTOR is a member of the PIKK superfamily that includes ATR, ATM, Mec1, and Tel1 proteins having homology to phosphatidylinositol lipid kinases. Evidence increasingly implicates mTOR as a central controller of cell growth and proliferation, and it controls initiation of translation of ribosomal proteins and several proteins that regulate cell cycle. Activation of ribosomal S6K1 after mitogen stimulation is dependent on mTOR (Chung et al. 1992; Kuo et al. 1992; Terada et al. 1992). Cap-dependent translation is facilitated by mTOR's phosphorylation and inactivation of 4E-BPs, suppressors of eukaryotic initiation factor 4E (eIF4E; Lin et al. 1994, Pause et al. 1994, Beretta et al. 1996). More recent findings have shown that mTOR may directly or indirectly control transcription, ribosomal biogenesis, actin cytoskeleton organization, and protein kinase C (reviewed in Schmelzle and Hall, 2000). Recently, our studies with rhabdomyosarcoma cells showed that activation of MAP kinases (p44/42) by growth factors was mTOR-dependent (Houghton et al. 2001; Harwood et al., manuscript submitted), implicating mTOR in cross-talk between the PI3K and MAPK pathways in some cell lines. Thus, the emerging picture places mTOR in a central role in which it senses mitogenic stimuli and amino acid (Iiboshi et al. 1999), ATP (Dennis et al. 2001), or nutrient (Rohde et al. 2001) conditions; mTOR coordinates many cellular processes related to growth and proliferation. The signaling pathways from IGF-IR to mTOR and downstream targets are depicted in Fig. 1.

## 5

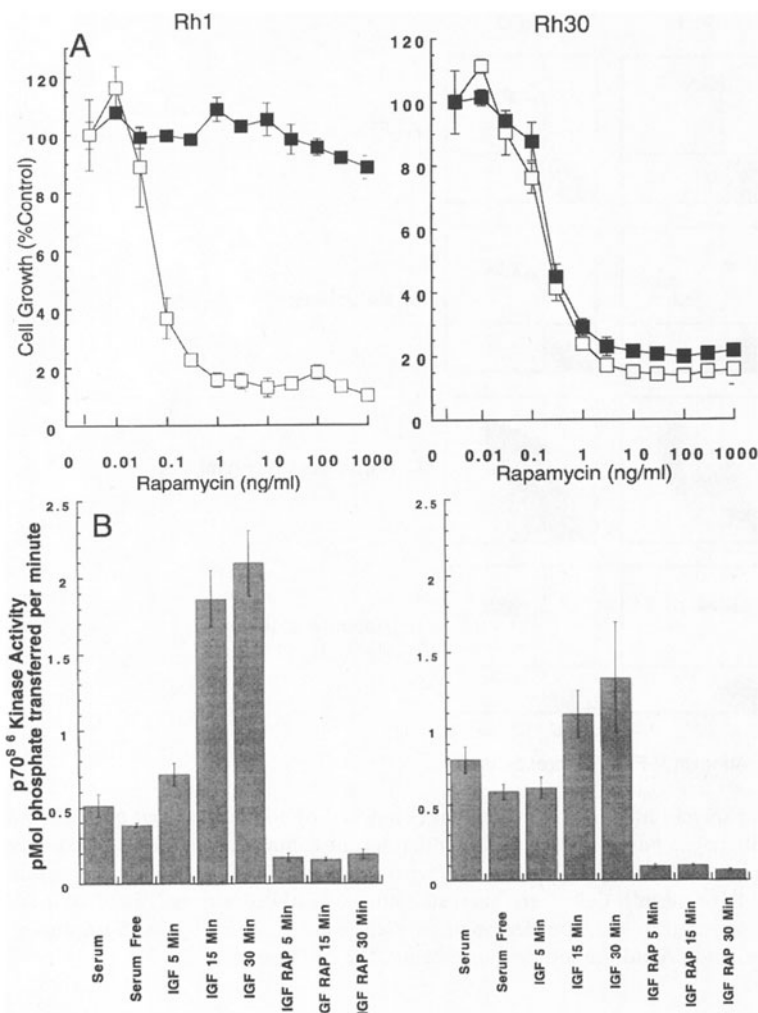
**Rapamycin Induces Apoptosis in Rhabdomyosarcoma Cells**

The basis for the differential sensitivity of Rh1 cells under serum-containing or serum-free conditions was of interest. The results suggested that some component of serum was able to rescue Rh1 cells but not Rh30 cells. Consequently, we attempted to identify components of serum

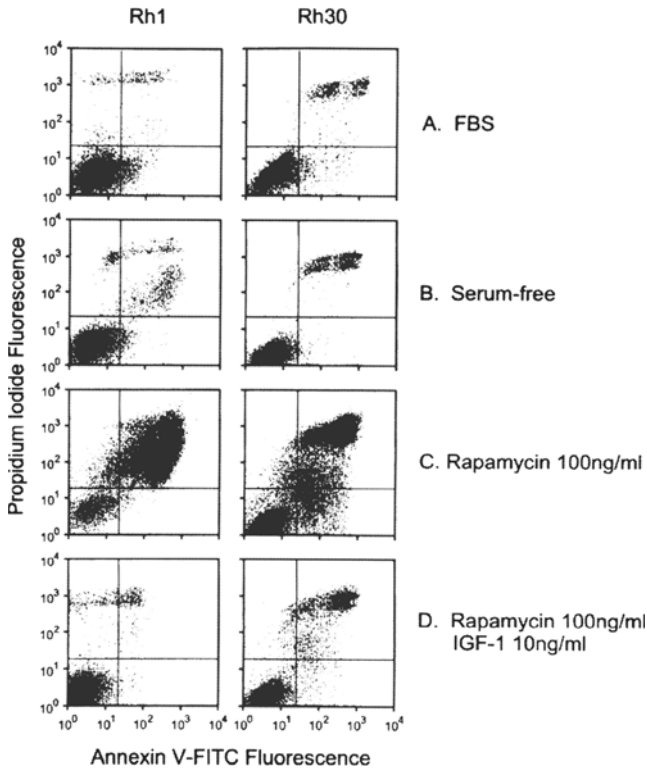


**Fig. 1** Simplified schema of signaling pathways from the IGF-I receptor to mTOR and Ras in mammalian cells

that protected from rapamycin (Hosoi et al. 1999). As shown in Fig. 2, under serum-free conditions IGF-I completely protected Rh1 cells, but had no apparent effect on the action of rapamycin against Rh30 cells. Further, IGF-I rescue was not a consequence of reversing rapamycin inhibition of ribosomal S6K1 activation, as S6K1 activity remained suppressed. So far the only growth factors shown to protect Rh1 cells are IGF-I, IGF-II, and to a lesser extent insulin (Thimmaiah et al. 2003). As IGF-I has been reported to prevent apoptosis in cells undergoing different forms of stress (Butt et al. 1999; Fujio et al. 2000; Kulik et al. 1997; Sell et al. 1995), we examined the fate of Rh1 and Rh30 cells treated under serum-free conditions with or without IGF-I supplementation. As shown in Fig. 3, under serum-free conditions rapamycin induces quite dramatic apoptosis of both Rh1 and Rh30 cells, and IGF-I essentially completely protects against the effect of rapamycin. Thus, under serum-free conditions the response to rapamycin is apoptosis, whereas under serum-containing conditions Rh30 cells are growth-arrested, but Rh1 cells continue to proliferate. Interestingly, expression of a rapamycin-resistant mTOR mutant (Ser2035→Ile) conferred resistance to both the growth-inhibiting and apoptosis-inducing activities of rapamycin. Thus,



**Fig. 2A, B** Inhibitory effect of rapamycin and effect of IGF-I on Rh1 (*left panels*) and Rh30 (*right panels*) rhabdomyosarcoma cells. **A** Rapamycin sensitivity. Cells were seeded and allowed to attach overnight, washed, and cultured in serum-free N2E in medium containing serial concentrations of rapamycin with (closed square) or without (open square) addition of IGF-I (10 ng/ml) for 7 days. Growth was assessed by lysing cells and counting nuclei. Results are presented as percent control. Cell number for control Rh1 cells was  $1.74 \times 10^5$ , and for Rh30 was  $5.7 \times 10^5$ , respectively. **B** Activation of p70<sup>S6</sup> K by IGF-I is blocked in Rh1 (*left panel*) and Rh30 (*right panel*) by rapamycin. Cells were serum-starved overnight, then stimulated with IGF-I (10 ng/ml) without or with preincubation for 15 min with rapamycin (100 ng/ml). Ribosomal p70<sup>S6</sup> K assays were performed on immunoprecipitates derived from  $3 \times 10^6$  cells. Each value represents the mean  $\pm$  SD for 4 determinations, and shows a representative experiment. (From Hosoi et al. 1999)



**Fig. 3** Rapamycin induces apoptosis that is blocked by IGF-I. Rh1 (*left panels*), and Rh30 cells (*right panels*) were grown under serum-containing (FBS) or serum-free (N2E) culture conditions supplemented with rapamycin (100 ng/ml) or rapamycin plus IGF-I (10 ng/ml). Cells were harvested after 6 (Rh1) or 4 days (Rh30), and apoptosis measured by the ApoAlert method. Cells were analyzed by FACS for annexin-V-FITC and propidium iodide fluorescence. (From Hosoi et al. 1999)

apoptosis is a consequence of inhibiting mTOR, and not through a second mechanism of action. Of note is that both Rh1 and Rh30 cells are mutant for the p53 tumor suppressor gene. This raised the possibility that p53 may be involved in sensing mTOR inhibition and cooperating in enforcing a G<sub>1</sub> arrest.

## 6

**The Tumor Suppressor p53 Protects from Rapamycin-Induced Apoptosis**

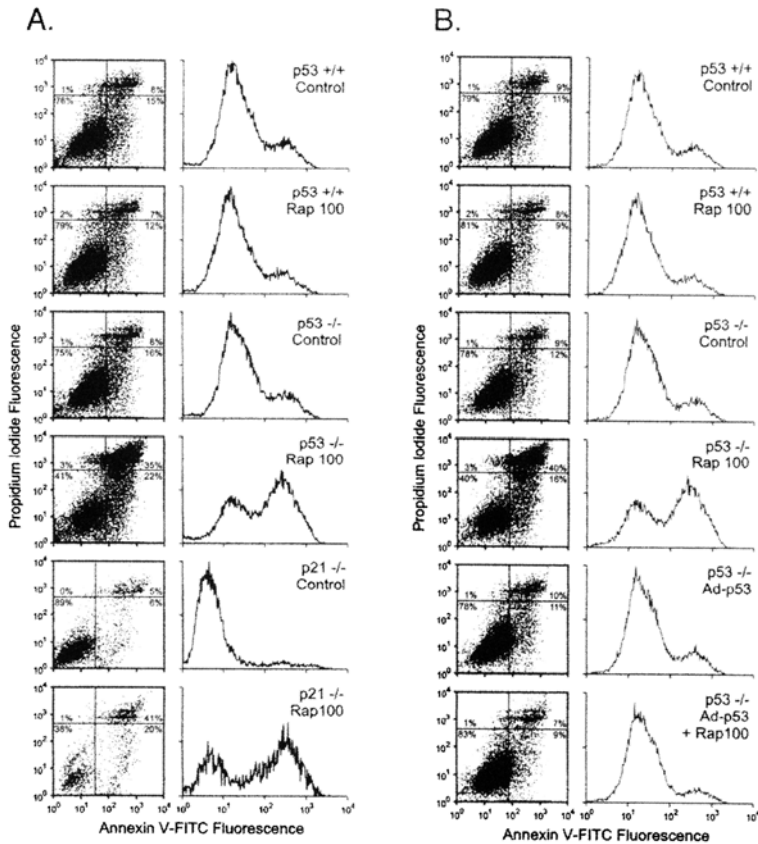
Cellular response to mTOR inhibition by rapamycin is generally characterized by p53-independent (Metcalf et al. 1997) cytostasis without cell death. In contrast, our studies have shown that the response to rapamycin in rhabdomyosarcoma cells lacking functional p53 is apoptosis (Hosoi et al. 1999; Huang et al. 2001). Apoptotic cells appeared to progress from G<sub>1</sub> to S phase, as >90% had incorporated BrdUrd in the presence of rapamycin. To determine whether cellular response to rapamycin was determined by p53 function, Rh30 cells were infected with adenoviruses expressing wild-type p53 or the cyclin-dependent kinase inhibitor p21<sup>Cip1</sup> (Huang et al. 2001). Expression of p53 or p21<sup>Cip1</sup> protected cells. We speculated that the mechanism was probably an enforcement of G<sub>1</sub> arrest and prevention of cells from initiating DNA replication. More recent data (Huang et al. 2003) suggest that cell cycle arrest is not the mechanism, as expression of a truncated p21<sup>Cip1</sup> allele lacking the nuclear localization signal does not cause G<sub>1</sub> accumulation, but protects against rapamycin-induced apoptosis.

To further investigate the role of p53 and p21<sup>Cip1</sup> in a non-transformed background we used murine embryo fibroblasts (MEFs) with disrupted p53 or p21<sup>Cip1</sup> genes. Specifically, MEFs with disruption of either p53 or p21<sup>Cip1</sup> undergo apoptosis whereas wild-type cells arrest in G<sub>1</sub> without loss of viability when treated with rapamycin (Fig. 4). Re-expression of wild-type p53 confers resistance to rapamycin-induced apoptosis (Huang et al. 2001). Exactly how mTOR signaling feeds into p53 remains to be determined. However, of importance for potential therapeutic application to cancer treatment is that rapamycin is selectively toxic to cells with attenuated p53-mediated G<sub>1</sub> checkpoint responses, at least in the absence of IGF-I.

## 7

**Mechanism(s) of Resistance to Rapamycin**

Rapamycin resistance was first found in yeast (Heitman et al. 1991). Mechanisms of rapamycin resistance are multiple and complicated; some of them have been identified and some remain to be elucidated. Cells may acquire resistance with or without mutagenesis. For instance,

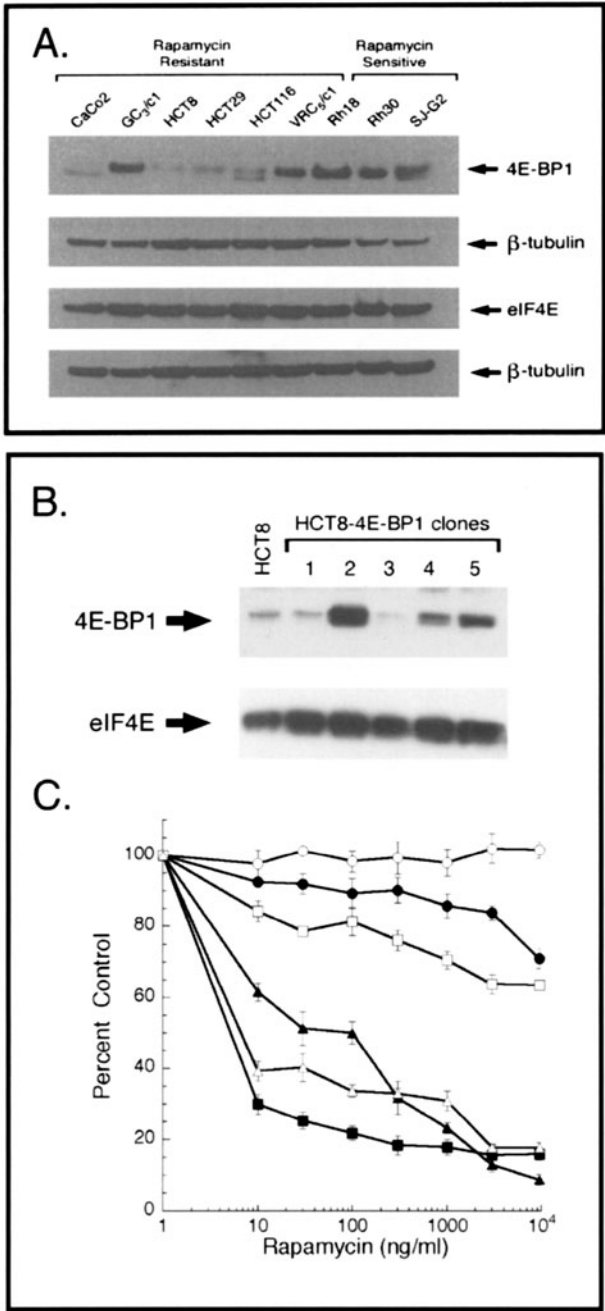


**Fig. 4** The cellular response to rapamycin is dependent on functional p53 or p21<sup>cip1</sup> in murine embryo fibroblasts. *Left:* Wild-type, p53<sup>-/-</sup>, and p21<sup>-/-</sup> MEFs were grown under serum-free conditions with or without rapamycin (100 ng/ml). After 5 days cells were harvested and apoptosis determined by ApoAlert assay. Results show a representative experiment. Percent distribution of cells in each quadrant is presented. *Right:* Wild-type, and p53<sup>-/-</sup> MEFs, and p53<sup>-/-</sup> MEFs infected with Ad-p53 (MOI of 100) were grown without or with rapamycin (100 ng/ml). Cells were harvested after 5 days and apoptosis determined by quantitative FACS analysis (ApoAlert), as described in Fig. 3. The percent distribution of cells in each quadrant is presented. Results show a representative experiment. (From Huang et al. 2001)



specific mutations in FKBP12 that prevent the formation of FKBP-rapamycin complex (Fruman et al. 1995), or certain mutations in FKBP-rapamycin-binding domain of mTOR (Ser2035→Ile) that block binding of FKBP-rapamycin complex to mTOR (Chen et al. 1995; Hosoi et al. 1998), conferred rapamycin resistance. Mutation of Thr389→Glu (Dennis et al. 1996) or Thr229→Ala or Glu (Sugiyama et al. 1996) of S6K1, a downstream effector of mTOR, also rendered S6K1 insensitivity to rapamycin.

As discussed in other chapters, the signaling pathway directly downstream of mTOR bifurcates (von Manteuffel et al. 1997). In mammalian cells, mTOR signals to both S6K1 and 4E-BP, and probably to other unidentified effectors. We were interested in understanding whether cellular responses to rapamycin (growth inhibition and apoptosis) were dictated by inhibition of signaling to either S6K1 or 4E-BP, or both. One approach to understanding which of these pathways is critical to cellular proliferation and survival was to select for rapamycin resistance and determine which pathway(s) altered to accommodate rapamycin inhibition of mTOR. Rapamycin-resistant clones (Rh30/Rapa10K) were obtained by continuously growing Rh30 cells in the presence of increasing concentrations of rapamycin, without prior mutagenesis (Hosoi et al. 1998). These initial studies showed that in resistant clones, rapamycin still inhibited IGF-I-stimulated mTOR-dependent activation of S6K1. However, of note, resistance was characterized by elevated c-MYC. Further independent clones were selected, either with or without mutagenesis (Dilling et al. 2002). Interestingly, resistance was unstable in each of the clones characterized. When the selecting pressure (i.e., rapamycin) was withdrawn, cells reverted to rapamycin sensitivity within 6–10 weeks. In resistant cells, as compared to parental cells, approximately tenfold less 4E-BP was bound to eIF4E, and total cellular 4E-BP was markedly reduced. In contrast, levels of eIF4E were unchanged. Steady-state levels of 4E-BP transcripts remained unaltered, but the rate of 4E-BP synthesis was reduced in resistant cells. Of importance, in cells that reverted to rapamycin sensitivity, levels of total 4E-BP returned to those of parental cells. Compared to parental cells, resistant clones had either similar or lower levels and activity of ribosomal S6K1, but c-MYC levels were elevated in both resistant and revertant clones. Further, anchorage-independent growth was enhanced in c-MYC-overexpressing cells, suggesting that the eIF4E pathway controls some aspects of the malignant phenotype (Lazaris-Karatzas et al. 1990; DeBenedetti et al. 1994). These results indicate that accommodation in the eukaryotic-initiation-factor 4E



(eIF4E) pathway occurs in rapamycin resistance through suppression of translation of 4E-BP. This suggests that inhibition of cap-dependent translation mediated by eIF4E is critical for rapamycin-induced growth arrest and apoptosis. Further, intrinsic or acquired resistance may be a consequence of low levels of 4E-BP suppressor proteins. To examine whether intrinsic resistance was associated with low 4E-BP, we examined levels in colon carcinoma cell lines previously characterized as rapamycin resistant (Dilling et al. 1994). Several of these cell lines with intrinsic rapamycin resistance were found to have low 4E-BP relative to eIF4E (Fig. 5A). To explore the role of 4E-BP in rapamycin resistance, stable clones of HCT8 colon carcinoma were engineered to overexpress 4E-BP. As shown in Fig. 5, rapamycin sensitivity increased markedly ( $>1,000$ -fold) as 4E-BP expression increased. These results suggest that the 4E-BP:eIF4E ratio is an important determinant of rapamycin resistance in some cell lines. However, this mechanism clearly does not account for resistance in other cell lines.

## 8

### Preclinical Antitumor Activity for Rapamycins

Initial in vivo testing of rapamycin as a potential antitumor agent was undertaken through the Division of Cancer Treatment at the National Cancer Institute (NCI; Douros and Suffness 1981; Houchens et al. 1983;



**Fig. 5A-C** Overexpression of 4E-BP abrogates resistance to rapamycin. **A** Western blot analysis of 4E-BP, eIF4E, and tubulin (loading control) in cell lines that have different intrinsic sensitivities to rapamycin. Colon carcinoma cell lines CaCo2, GC<sub>3</sub>/c1, HCT8, HCT29, HCT116, and VRC5/c1 are intrinsically resistant to rapamycin with IC<sub>50</sub> concentrations  $>1,200$  ng/ml. Pediatric solid tumor lines SJ-G2 (glioblastoma) and Rh18 and Rh30 (rhabdomyosarcoma) are sensitive to rapamycin (IC<sub>50</sub>  $<1$  ng/ml). **B** Expression of 4E-BP and eIF4E in HCT8 clones stably transfected with a 4E-BP expression plasmid (pcDNA3-PHAS-I). Expression of 4E-BP was greater in clones C2, C4, and C5 than in parental HCT8 cells, but expression of was similar in parental and C1 and C3 transfected clones. **C** Sensitivity to rapamycin. Cells were plated at low density in increasing concentrations of rapamycin, and colonies were counted after 7 days of exposure to rapamycin. Symbols: Parental HCT8 (closed circle) and clones C1 (open circle), C2 (closed square), C3 (open square), C4 (closed triangle), and C5 (open triangle). Each *point* is the mean $\pm$ SD of three determinations. (From Dilling et al. 2002)

Eng et al. 1984). These preliminary results revealed that rapamycin significantly inhibited the growth of syngeneic B16 melanoma, colon carcinoma models 26 and 38 tumor, CD8F1 mammary tumor, and EM ependymoblastoma. Rapamycin, in the active dose range, was less toxic than conventional antitumor drugs, such as 5-fluorouracil, cyclophosphamide, and adriamycin (Eng et al. 1984). However, at that time Ayerst Research Laboratories (Montreal, Canada), where rapamycin was first isolated and characterized, abandoned rapamycin as an antitumor agent because they failed to develop a satisfactory intravenous formulation for clinical trials. Recently, two rapamycin ester analogues, CCI-779 [rapamycin-42, 2, 2-bis(hydroxymethyl)-propionic acid; Wyeth-Ayerst] and RAD001 [everolimus, 40-O-(2-hydroxyethyl)-rapamycin; Novartis, Basel, Switzerland], with improved pharmaceutical properties have been synthesized and evaluated. CCI-779 is designed for intravenous injection, whereas RAD001 for oral administration. Both have antitumor effects similar to rapamycin, are currently being developed as antitumor agents, and are undergoing phase I–III clinical trials. In culture, rapamycin and CCI-779 potently inhibit growth of numerous malignant cell lines, including those derived from rhabdomyosarcoma, neuroblastoma, glioblastoma, medulloblastoma, small cell lung cancer (Dilling et al. 1994; Shi et al. 1995; Seufferlein and Rozengurt, 1996; Hosoi et al. 1998; Georger et al. 2001; Dudkin et al. 2001), osteosarcoma (Ogawa et al. 1998), pancreatic carcinoma (Grewe et al. 1999; Shah et al. 2001), breast and prostate carcinoma (Gibbons et al. 2000; Yu et al. 2001), murine melanoma, T-cell leukemia, and B-cell lymphoma (Houchens et al. 1983; Hultsch et al. 1992; Gottschalk et al. 1994; Muthukkumar et al. 1995). CCI-779 *in vivo* also inhibited the growth of human U251 malignant glioma cells that were resistant to rapamycin *in vitro* (Georger et al. 2001). CCI-779 induced tumor growth inhibition correlated with decreased phosphorylation of 4E-BP (Dudkin et al. 2001).

## 9

### Clinical Trials for Antitumor Efficacy of Rapamycins

As alluded to, RAD001 and CCI-779 are undergoing phase I and phases II/III clinical trials, respectively. However, only preliminary results from phase I trials of CCI-779 are currently available. Two groups conducted phase I trials for CCI-779 using different schedules (Hidalgo et al. 2000; Raymond et al. 2000). In the USA, CCI-779 was administered as a 30-

min intravenous infusion daily for 5 days every 2 weeks at doses of 15~24 mg/m<sup>2</sup> per day. Of 45 patients with various types of cancer, nine of them showed some evidence of tumor response (Hidalgo et al. 2000). In Europe, CCI-779 was administered as a 30-min intravenous infusion weekly at doses of 7.5~220 mg/m<sup>2</sup> per week (Raymond et al. 2000). After  $\geq 8$  weekly doses, of 18 patients, significant tumor regressions were observed in two patients with lung metastasis of renal cell carcinomas (both treated with 15 mg/m<sup>2</sup> per week) and in one patient with a neuroendocrine tumor of the lung treated with 22.5 mg/m<sup>2</sup> per week. Two patients experienced tumor stabilization. Consistently, these two groups showed that CCI-779 was well tolerated in patients with only mild side effects, such as acneform rash, mild mucositis, some thrombocytopenia, and elevated triglyceride and cholesterol levels (Hidalgo and Rowinsky 2000). It is anticipated that these agents will be evaluated against additional histiotypes in phase II trials.

10

## Conclusions

Rapamycin and its derivatives represent novel agents for therapy of human cancer. In this review we have detailed some of the studies from this and other laboratories that have assisted in development of this class of drug specifically as cancer chemotherapeutic agents. Other studies, many reported in this book, have elucidated the mechanism of action of rapamycin, and have identified pathways proximal and distal to mTOR that have lead to a greater understanding of how this class of drug exerts cytostatic activity. Our studies have suggested that cellular response to rapamycin is converted from G<sub>1</sub> cytostasis to apoptosis when p53 is mutated in tumor cells, or disrupted in embryo fibroblasts. These results suggest that combining rapamycin, or its derivatives, with an inhibitor of the IGF-I receptor may induce apoptosis selectively in tumor cells with mutated p53. As both humanized antibodies against this receptor and small molecule inhibitors are in development, the validity of this approach will be amenable to testing relatively quickly. Recently, Neshat et al. (2001) have shown that PTEN (phosphatase and tensin homolog deleted on chromosome ten)-mutated or PTEN-deficient tumor cells were more sensitive to CCI-779 (Podsypanina et al. 2001; Neshat et al. 2001). The PTEN-mutated tumor cells demonstrated elevated levels of phosphorylated Akt and activated S6K1 (Podsypanina et al. 2001). Loss

of PTEN by deletion or mutation has been reported in approximately 50% of all solid human tumors (Simpson and Parsons 2001). Thus, there are at least two mechanisms by which to anticipate tumor-selective activity of rapamycin analogues as cancer chemotherapeutic agents.

*Acknowledgment.* We wish to dedicate this review to the memory of Charles B. Pratt, M.D., who dedicated his life to helping children with cancer at St. Jude and throughout the world. Studies from this laboratory were supported by USPHS awards CA23099, CA77776, CA96696 and CA21765.

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